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Targeting Inhibition of Fibroblast Activation Protein-α and Prolyl Oligopeptidase Activities on Cells Common to Metastatic Tumor Microenvironments[1]

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Abstract

Fibroblast activation protein (FAP), a membrane prolyl-specific proteinase with both dipeptidase and endopeptidase activities, is overexpressed by reactive stromal fibroblasts during epithelial-derived cancer growth. FAP digests extracellular matrix as tissue is remodeled during cancer expansion and may also promote an immunotolerant tumor microenvironment. Recent studies suggest that nonspecific FAP inhibitors suppress human cancer xenografts in mouse models. Prolyl oligopeptidase (POP), another prolyl-specific serine proteinase, is also elevated in many cancers and may have a regulatory role in angiogenesis promotion. FAP and POP cell–associated activities may be targets for diagnosis and treatment of various cancers, but their accessibilities to highly effective specific inhibitors have not been shown for cells important to cancer growth. Despite their frequent simultaneous expression in many cancers and their overlapping activities toward commonly used substrates, precise, separate measurement of FAP or POP activity has largely been ignored. To distinguish each of the two activities, we synthesized highly specific substrates and inhibitors for FAP or POP based on amino acid sequences surrounding the scissile bonds of their respective putative substrates. We found varying amounts of FAP and POP protein and activities on activated fibroblasts, mesenchymal cells, normal breast cells, and one breast cancer cell line, with some cells exhibiting more POP than FAP activity. Replicating endothelial cells (ECs) expressed POP but not FAP until tubulogenesis began. Targeting FAP-positive cells, especially mesenchymal stem cells and cancer-associated fibroblasts for inactivation or destruction, and inhibiting POP-producing EC may abrogate stromal invasion and angiogenesis simultaneously and thereby diminish cancer growth.

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Introduction

As a primary malignancy invades surrounding tissues or metastasizes to distal sites, even tumor cell growths of 1- to 2-mm diameter require a stromal microenvironment composed of activated fibroblasts, endothelial cells (ECs) involved in tubulogenesis, and extracellular matrix (ECM) that is constantly remodeled to accommodate growth. In addition, precursor mesenchymal stem cells (MSCs), their putative derivative cancer-associated fibroblasts, and cancer stem cells may also be present. The prolyl-specific serine proteinase, fibroblast activation protein (FAP), a type II integral membrane protein, is regularly overexpressed on the stroma of >90% of epithelial-derived cancers and their metastases [1–3]. FAP is produced transiently by activated stromal fibroblasts during embryogenesis [4], the latter stages of wound healing

Abbreviations: AEEA, 2-(2-(2-aminoethoxy)ethoxy)acetic acid; APCE, antiplasmin-cleaving enzyme; C95, acetyl-Arg-AEEA-Gly-Pro-AMC (fluorescent FAP/POP substrate); FAP, fibroblast activation protein; HCCs, HCC1419 breast cancer cells; HMVECd, human dermal microvascular endothelial cells; J94, acetyl-Lys-Leu-Arg-(L)boroPro (POP inhibitor); M83, acetyl-Arg-AEEA-(D)Ala-(L)boroPro (POP and FAP inhibitor); MCF-12A, MCF12A normal breast cells; MDA, MDA-MB436 breast cancer cells; MSCs, mesenchymal stem cells; POP, prolyl oligopeptidase; VA-13, WI-38 VA-13 2RA (SV-40 viral-transformed fibroblast cells); WI-38, fibroblast cells

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[3], in certain pathologic states in which fibrous tissue growth is a conspicuous feature [5–9], and occasionally on normal fibroblast or pancreatic α-cells. FAP is not characteristically found on normal tissues or benign tumors [2,3,10]. Taken together, these observations prompted the suggestion that FAP may carry powerful potential as an ideal therapeutic target in a number of cancers [11–14].

The *in vivo* function of membrane-inserted FAP remains poorly understood, likely because a biologic substrate for its proteinase activity has not been definitively established; however, reports that FAP cleaves gelatin [2,15,16] and partially denatured or degraded type I collagen [17,18] suggest that FAP helps digest ECM components as tissue is remodeled to accommodate cancer expansion [2,19,20]. Paradoxically, activated fibroblasts not only digest ECM but also synthesize ECM components of the stromal scaffolding that support cell division and motility during neoplastic growth [21]. FAP proteolytic activity has been considered the most obvious useful property to target for inhibition when designing new therapeutic approaches to the large number of FAP-containing cancers [11,12]. Santos et al. [22] have shown that genetic deletion or pharmacologic inhibition of FAP by glutamyl-proline boronic acid (Glu-boroPro) decreased stromal growth in mouse models of lung and colon cancer. Unfortunately, however, Glu-boroPro has an exceptionally short plasma half-life before cyclizing and losing inhibitory activity [23]. Moreover, it also inhibits dipeptidyl peptidase IV, which is important in plasma glucose regulation and immune function [24]. Hence, despite inhibiting FAP and suppressing tumor growth, Glu-boroPro is not likely to be therapeutically useful in cancer [25].

The accessibility and measurement of cell membrane FAP activity and its inhibition remains incompletely studied, particularly with respect to the different cells commonly found in tumor microenvironments. Additionally, although not always appreciated, the measurement of FAP activity is confounded by another prolyl endopeptidase, namely, prolyl oligopeptidase (POP), which is expressed by a number of normal cell types and is commonly elevated in many cancers [26]. Recently, POP has been suggested to make secondary cleavages in partially degraded thymosin-β_4 to yield the derivative peptide, acetyl-SDKP, which appears to be a potent stimulator of angiogenesis [27]. Both FAP and POP activities are regularly measured using nonspecific substrates such as Z-Gly-Pro-AMC or succinyl-Gly-Pro-AMC, neither of which distinguishes between the two activities [28]. Consequently, total prolyl-specific endopeptidase activity, which is often attributed to FAP alone, may also include POP activity and thereby complicate interpretations about the effects of inhibiting either enzyme on cancer growth, particularly since both enzymes appear commonly overexpressed by several cell types that comprise metastatic tumor microenvironments.

Our discovery of antiplasmin-cleaving enzyme (APCE) in human plasma and its virtual identity with FAP has made APCE a useful FAP surrogate for constructing highly specific and sensitive low molecular weight pseudo-peptide substrates and inhibitors for APCE or FAP [15,28–30]. Previously published preferred substrate peptide sequences for cleavage by POP [31] proved valuable for the design and synthesis of a specific and sensitive inhibitor of POP. We now report measurements of membrane-associated FAP and POP activities over time and show that each can be effectively inhibited while on live cells that typify microenvironments of expanding and metastasizing tumor niches, namely, MSCs, activated fibroblasts, two breast cancer cell lines, and ECs. Our results suggest that the two proteinases may be synergistic for tumor growth. Either enzyme is a potential target for rapid, specific inhibition by pseudo-peptide inhibitors that were designed to be coherent with active site structural features and thereby present unique therapeutic potential for treatment of epithelial-derived malignancies.

Materials and Methods

Cell Culture

Fibroblasts, WI-38 and WI-38 VA13 2RA (VA-13), breast cancer cells, MDA-MB436 (MDA) and HCC1419 (HCCs), and normal breast cells, MCF-12A, were all purchased from American Type Culture Collection (ATCC, Manassas, VA). Human dermal microvascular endothelial cells (HMVECd) and MSCs were purchased from Lonza (Walkersville, MD). All cells were authenticated by the companies and used within 6 months of purchase or recovery from cryopreservation. WI-38 and VA-13 were grown in minimum essential medium (Mediatech Inc, Manassas, VA) supplemented with 10% fetal calf serum (Gibco, Grand Island, NY), 2 mM GlutaMAX-1 (Gibco), and 1 mM sodium pyruvate (Gibco). MDA and HCCs were grown in Dulbecco's modified Eagle's medium (DMEM; Mediatech Inc) supplemented with 10% fetal calf serum. MCF-12A and HMVECd were grown in EGM2-MV (Lonza) with all provided supplements, unless otherwise specified. MSCs were grown in MSCGM (Lonza).

Monoclonal Antibody Production

Human APCE was purified as previously described [29] and used as the antigen for monoclonal antibody (mAb) production. The hybridoma cells were produced at the Hybridoma Center for Agricultural and Biological Sciences at Oklahoma State University. After initial screening against pure APCE by direct ELISA, the positive cells were cloned three times by limiting dilution. Selected antibodies were produced in serum-free media (Gibco) in roller bottles, then purified using MEP HyperCel (Pall, Port Washington, NY) chromatography and isotyped using the Pierce Rapid Isotyping Kit (Thermo Scientific, Waltham, MA). Of 24 mAbs identified, mAb 6D2, an IgG1κ antibody, recognized both APCE and FAP by ELISA or Western blot analysis with the greatest sensitivity and was used in these studies. Mouse F19 mAb to FAP was produced and purified from cultures of hybridoma cells purchased from ATCC and used for confocal imaging and immunoprecipitation (IP).

Immunostaining

Selected normal or neoplastic cells were grown to confluency in 10-cm tissue culture dishes, rinsed in phosphate-buffered saline (PBS), and lysed on the plate in 1 ml of 2× Laemmli denaturing sample buffer with DTT for whole-cell lysates. Membrane and cytosolic fractions were prepared from confluent tissue culture dishes using the Mem-PER Kit (Pierce) as per the manufacturer's instructions. Samples were electrophoresed under reducing conditions on 4% to 12% Bis-tris sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels (Invitrogen, Grand Island, NY) and transferred to nitrocellulose for Western blot analysis. After blocking with 3% BSA/TBS-Tween (TBST), blots were incubated with a combination of 0.5 μg/ml mAb 6D2 (anti-FAP), 0.1 μg/ml anti–α-tubulin (Sigma, St Louis, MO; #6199), and 0.1 μg/ml anti-actin (Abcam, Cambridge, MA; 2Q1055), in 1% BSA/TBST, and then washed and incubated with 1:60,000 goat anti–mouse HRP (Thermo Fisher, Pittsburgh, PA). Blots for POP were blocked with 3% BSA/TBST, then incubated with a combination of 0.1 μg/ml goat anti-POP (R&D Systems, Minneapolis, MN; #AF4308), 0.1 μg/ml anti–α-tubulin (Sigma;

#6199), and 0.1 μg/ml anti-actin (Abcam; 2Q1055) in 1.5% BSA/0.5 M NaCl/TBST. After washing in the same buffer, blots were incubated with 1:18,000 rabbit anti–goat HRP (R&D Systems; #HAF017) in 1.5% BSA/0.5 M NaCl/TBST. ECL-Plus (Thermo Fisher) was added and blots were visualized on RPI blue radiographic film.

Confocal analysis of FAP protein in cells was accomplished first by growing cells to confluence on four-well glass chamber slides (Lab-Tek, Waltham, MN). The cells were fixed with 0.75% paraformaldehyde, blocked with 1% BSA/PBS, and then incubated with mouse F19 mAb (2 μg/ml) or isotype control antibody, MOPC 21 (Sigma; 2 μg/ml) in 0.1% BSA/PBS, with 0.1% saponin. After washing, the slides were incubated with goat anti–mouse Alexa Fluor 568 (Invitrogen) at 1:2000 in 0.1% BSA/PBS and then mounted with Prolong Gold/4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Grand Island, NY). Cells were visualized using a Leica TCS NT Microscope fitted with a 40× Plan Fluotar 1.0 NA oil immersion objective. Images were analyzed using Leica TCS and Volocity software.

Protein Characterization

Cells of each type were grown to confluence in 10-cm tissue culture dishes, rinsed in PBS, and then lysed in 1 ml of ice-cold IP buffer containing 1% Triton/150 mM NaCl/10 mM Tris (pH 7.5)/1 mM EDTA/1 mM EGTA/0.5% NP-40/10% sucrose with cOmplete ULTRA protease inhibitor cocktail (Roche, Indianapolis, IN) added. Whole-cell lysates were centrifuged to remove detergent-insoluble proteins. mAb F19 (5 μg/ml) was added and allowed to bind overnight at 4°C. Then, 25 μl of 75% slurry of Protein G beads (Amersham, Pittsburgh, PA) in TBS was added and incubated for 1 hour at 4°C. Protein G beads were spun down, washed three times with IP buffer, resuspended in loading buffer, and boiled for 5 minutes; the beads were removed by centrifugation and a portion of the supernatant was electrophoresed under reducing conditions on 4% to 12% Bis-tris SDS-PAGE gels. To confirm the presence of FAP, the regions of each lane corresponding to the molecular weight of FAP were excised and the proteins within each gel slice were reduced with tris[2-carboxyethyl]phosphine, then alkylated with iodoacetamide, and digested with trypsin as described by the In-gel Tryptic Digestion Kit protocol (Thermo Fisher). Each trypsin digest sample was analyzed by high-performance liquid chromatography–tandem mass spectrometry (LC/MS/MS) on a nanoscale Dionex UltiMate 3000 HPLC equipped with an Acclaim PepMap C18 column (75-μm internal diameter × 15-cm length with 3-μm particles) connected to an AB-Sciex QSTAR Elite mass spectrometer. The peptide molecular weights and MS/MS fragment ion spectra observed for each peptide were used to query an National Center for Biotechnology Information comprehensive nonidentical human protein database (updated 31 January 2011) loaded on an in-house MASCOT database server (version 2.3).

Inhibitors and Substrate

The pseudo-peptide inhibitor, acetyl-Arg-AEEA-(D)Ala-(L)boroPro [AEEA, 2-(2-(2-aminoethoxy)ethoxy)acetic acid], M83, with dual FAP (K_i = 5.7 nM) and POP (K_i = 7.4 nM) inhibition; the specific POP inhibitor, acetyl-Lys-Leu-Arg-(L)boroPro, J94 (K_i < 100 nM); and the substrate for both FAP and POP, acetyl-Arg-AEEA-Gly-Pro-AMC, C95, were designed, synthesized, and characterized by us as previously reported [28]; all are soluble in aqueous buffers. The inhibitors (J94 and M83) were dissolved in Hank's balanced salt solution (HBSS; Gibco) at 1 mM and stored at −20°C. The substrate (C95) was dissolved in HBSS at 2.5 mM and stored at 4°C. FAP and POP at equimolar concentrations cleaved C95 substrate at equivalent rates (data not shown).

Whole-cell Activity Assays

Cells were cultured in normal growth media unless otherwise stated. For cells grown in media with selected components either withheld or added, and depending on each experimental design as indicated in the Results section, growth times were chosen from 7 to 14 days in specific media before plating for activity assays. For activity assays, cells were plated onto 96-well black-sided, clear bottom tissue culture plates (Costar) at densities selected for achieving confluency in 3 days. Cell densities varied with cell type and the selected growth media; the number of cells necessary for plating was estimated from pilot experiments. After 3 days of growth, cultures were washed with HBSS. The wash solution was replaced with 188 μl of fresh buffer (HBSS) and 2 μl of either buffer (Control) or M83 or J94 inhibitor (10 μM final) was added. Ten microliters of fluorescent substrate, C95, was added to give a final concentration of 125 μM, and fluorescence was measured with time at 360/460 nm excitation/emission wavelengths using an FL600 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT). Fluorescence units were converted to FAP units/100,000 cells by using a conversion factor determined from an APCE standard curve of prolyl-specific endopeptidase activity, such that 1 FAP unit is equivalent to the fluorescence produced by 1 ng of APCE/min. Since FAP and POP cleave the C95 substrate at an equivalent rate, both FAP and POP activities are expressed as FAP units in all figures.

For tube formation assays, Matrigel (BD Biosciences, San Jose, CA; 80 μl) was added to black-sided, clear bottom 96-well plates as above and allowed to gel at 37°C for 30 minutes. Then, 15,000 HMVECd in EC medium without serum were added and allowed to settle for 1 hour. Either buffer, the M83 inhibitor, or the J94 inhibitor (10 μM final) was added, and the assay then started by adding the dual FAP/POP fluorescent C95 substrate. The reader was maintained at 37°C and fluorescence was measured with time for 18 hours.

Results

FAP/APCE and POP are prolyl-specific serine endopeptidases, with the proteolytic activity of POP restricted to peptides less than about 30 residues [32]. FAP is membrane-inserted and POP is membrane-associated [33,34], while APCE circulates in blood, possibly as a shed-soluble derivative or splice variant of FAP [15]. Nonspecific dipeptides containing prolyl boronic acid compounds or mutations of the serine active site in FAP inhibit FAP proteolytic activity on cancer-associated fibroblasts and diminishes cancer growth [20,22]. To date, the accessibility and rapidity by which inhibitors block activity of membrane-bound FAP have not been directly assessed. POP, commonly expressed by neoplasms [6,26,34–36], manifests overlapping proteolytic activity with FAP/APCE when measured as usual with nonspecific fluorescent peptide substrates such as Z-Gly-Pro-AMC or succinyl-Gly-Pro-AMC [28]. Efforts to use inhibitors to separate the two enzymatic activities have likewise been compromised by significant inhibition of both FAP and POP [28]. The problem of measuring FAP and POP activities separately in biologic or pathologic scenarios where either may play a critical role has received scant attention, which we now address through the use of specific FAP and POP substrates and inhibitors designed and characterized in our laboratory [28].

In Figure 1*A*, confocal microscopy of immunostained saponin-permeabilized WI-38 fibroblasts shows abundant FAP, most of which appears membrane-associated. As reported, FAP was absent in SV-40 transformed VA-13 fibroblasts [10]. Figure 1*B* shows results for FAP and POP activity assays, using highly selective, specific substrates and inhibitors we previously described [28]. Overlying medium was first removed from each cell culture after which cells were washed with physiologic buffer and overlaid with fresh buffer. FAP or POP inhibitor was added, and each culture assayed with time for both FAP and POP activities. By using the specific substrate, C95, that is cleaved only by FAP or POP in conjunction with the high affinity POP inhibitor, J94, that has strict specificity for POP, but none toward FAP, we were able to measure each enzyme's activity [28]. For each cell type, total prolyl-specific endopeptidase activity on intact cell membranes was determined using C95 substrate, which is cleaved at equal rates by FAP or POP; on parallel cultures, J94 inhibitor was added to block solely that activity due to POP, thereby defining the remaining endopeptidase activity as uniquely attributable to membrane-associated FAP (Figures 1*B*, 3, 4*B*, 5*B*, 6, and 8*B*). As expected, the dipeptidase, DPPIV, neither cleaved C95 substrate nor was it inhibited by M83 or J94. Our previous studies showed that APCE and FAP have essentially identical activities [15], thereby allowing the use of an APCE standard curve to determine moles of active FAP on the cell surface and, hence, the number of FAP proteinase molecules per cell. Therefore, knowing the FAP activity and number of WI-38 fibroblasts in a confluent well, each cell was estimated to have ~117,000 FAP molecules on its membrane surface. Figure 1*C* demonstrates FAP by Western blot and Figure 1*D* indicates that the majority of FAP is membrane-associated, with a lesser amount in the cytosol, which agrees with our confocal results. Figure 1*E* shows POP by Western blot in both WI-38 and VA-13 fibroblasts, although the activity assay did not detect any surface accessible POP activity in VA-13 cells. The majority of POP is cytosolic with a significant fraction being membrane-associated, which agrees with our assay results of cell surface POP activity (Figure 1*F*).

Neither FAP nor POP activity was found in media that had been in contact with cells for 3 days, indicating that neither enzyme was shed or released (data not shown). When fresh buffer was again placed over the cells, both FAP and POP activities were found to be unchanged and immediately detectable, thereby supporting the membrane location of both enzymes. As can be seen in Figure 1*B*, WI-38 fibroblasts yielded higher FAP than POP activity. In Figure 1*C*, immunoblot analyses of lysates derived from a specified number of cells of each cell type, using the cellular content of α-tubulin (50 kDa) and actin (43 kDa) as protein load controls, showed relative FAP levels in accord with those estimated by confocal microscopy of corresponding immunostained cells or by endopeptidase activity measurements. As shown in Figure 2, amino acid sequence determinations for tryptic peptides of the isolated putative FAP protein band from each cell type ensured that the ~100 kDa band from WI-38 fibroblasts was indeed FAP.

Figure 3 illustrates the effectiveness and rapidity of FAP and POP inhibition by the pseudo-peptide inhibitor construct, M83. Cultured W-I38 cells were exposed to the inhibitor at either the beginning of

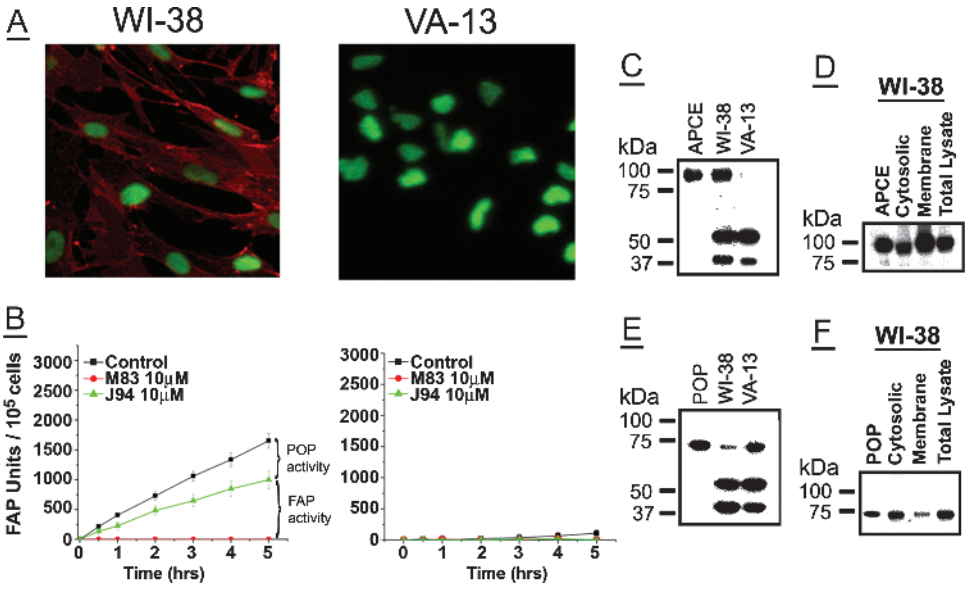

Figure 1. Characterization of FAP and POP in fibroblasts. (A) Confocal images of permeabilized WI-38 and VA-13 fibroblasts grown on plastic slides and labeled with mouse anti-FAP mAb F19 followed by anti–mouse Alexa Fluor 568 (red) and the DNA stain DAPI (green). (B) FAP and POP activities of fibroblasts grown on plastic wells as measured by cleavage of fluorescent substrate C95 and using a POP specific inhibitor J94 to separate the two activities. One FAP unit = Δfluorescence/min on cleavage of C95 by 1 ng of APCE. (C) Immunostains of cell lysates from fibroblast cultures using mouse anti-FAP mAb 6D2. One nanogram of APCE served as a positive control, while intracellular contents of α-tubulin (50 kDa) and actin (43 kDa) were used for standardizing the amount of cell lysate protein applied in each lane. (D) Immunostains of cell lysates and membrane and cytosol fractions from WI-38 fibroblast cultures using mouse anti-FAP mAb 6D2. One nanogram of APCE served as a positive control. (E) Immunostains of cell lysates from fibroblast cultures using goat anti-POP. One nanogram of POP served as a positive control, while α-tubulin (50 kDa) and actin (43 kDa) were used for standardizing loads. (F) Immunostains of cell lysates and membrane and cytosol fractions from WI-38 fibroblast cultures using goat anti-POP. One nanogram of POP served as a positive control.

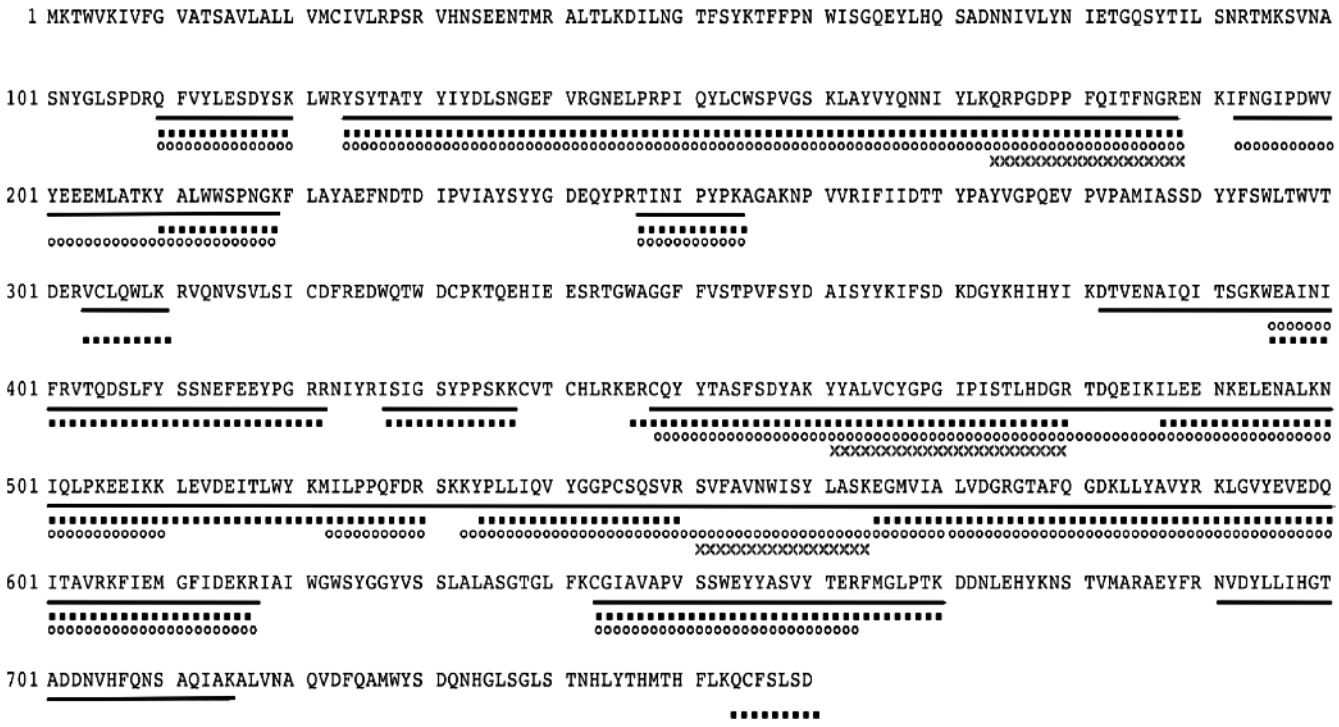

Figure 2. Amino acid sequence coverage for FAP isolated from four human cell types. Protein was purified by immunoprecipitation and SDS-PAGE followed by sequence determination by LC/MS/MS; human MSCs (———), 51% (coverage FAP sequence); WI-38 fibroblasts (■ ■ ■ ■), 42%; MDA (○○○○), 41%; and HMVECd deprived of hydrocortisone (⊠⊠⊠), 6.4%. In each case, the FAP-containing region of the gel was located by reference to a 100-kDa protein standard band in an adjacent lane.

the assay or after 2 hours of incubation with the C95 substrate that is cleaved by FAP or POP. After an increasing fluorescent signal indicated the accrual of significant proteolytic activity, the addition of even nanomolar concentrations of the M83 inhibitor instantly abolished endopeptidase activity as evidenced by lack of further fluorescence increase.

As shown in Figure 4, three methods of assessment, namely, confocal microscopy, Western blot analysis, and high specificity endopeptidase activity, indicated that human MCF-12A or human MDA contained far less FAP protein and FAP activity than observed for WI-38 activated fibroblasts (Figure 1). While POP activity for MCF-12A cells and WI-38 fibroblasts was similar, human MDA had about five times that amount. Whole-cell lysates of MCF-12A, MDA, and HCCs were subjected to Western blot analysis, which demonstrated an approximately equal amount of POP in all cells (Figure 4*D*). MDA had greater POP activity than the other two cell types, but the assay only measures activity of cell surface POP and not intracellular enzyme. HCCs contained neither FAP protein nor FAP activity. To date, overexpression of POP in neoplasms remains unexplained, but recent results of Myohanen et al. [37] suggest that POP may be responsible for a second-step proteolytic cleavage in the autoregulation of thymosin-β_4 that yields the derivative tetrapeptide, acetyl-SDKP, a potent stimulator of angiogenesis.

In Figure 5*A*, confocal microscopy of cultured normal HMVECd on plastic demonstrated that cell-associated FAP was only occasionally encountered (the figure shows a field containing a rare positive cell). Western blot analysis of HMVECd lysates lacked a band consistent with FAP protein (Figure 5*C*). Likewise, HMVECd were devoid of FAP activity (Figure 5*B*), but HMVECd cultures did contain considerable POP activity and the presence of the protein was confirmed by Western blot (Figure 5*D*). However, as shown in Figure 6*B*, when grown on Matrigel and allowed to form tubules over an 18-hour period, significant amounts of both FAP and POP activities were expressed and easily detectable. POP expression began just before capillary-like tubules started forming and continued

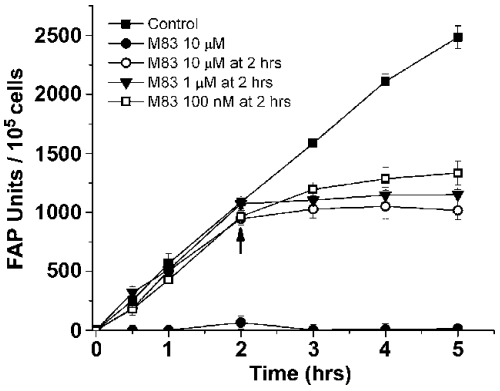

Figure 3. Dose-response inhibition of FAP activity on WI-38 fibroblast surfaces by M83. WI-38 fibroblasts were grown on plastic and washed once with HBSS, after which the M83 inhibitor (10 μM) in HBSS was added at zero time to one set and buffer only to the other four sets. The fluorescent substrate C95 was added to all sets of wells and Δfluorescence/min, reflecting cleavage of C95 over time, was allowed to proceed for 2 hours when 10 μM, 1 μM, or 100 nM of the M83 inhibitor was added to each of three sets of wells. As shown, each inhibitor concentration instantly, and essentially totally, inhibited the proteolytic activity of FAP on the cell surface.

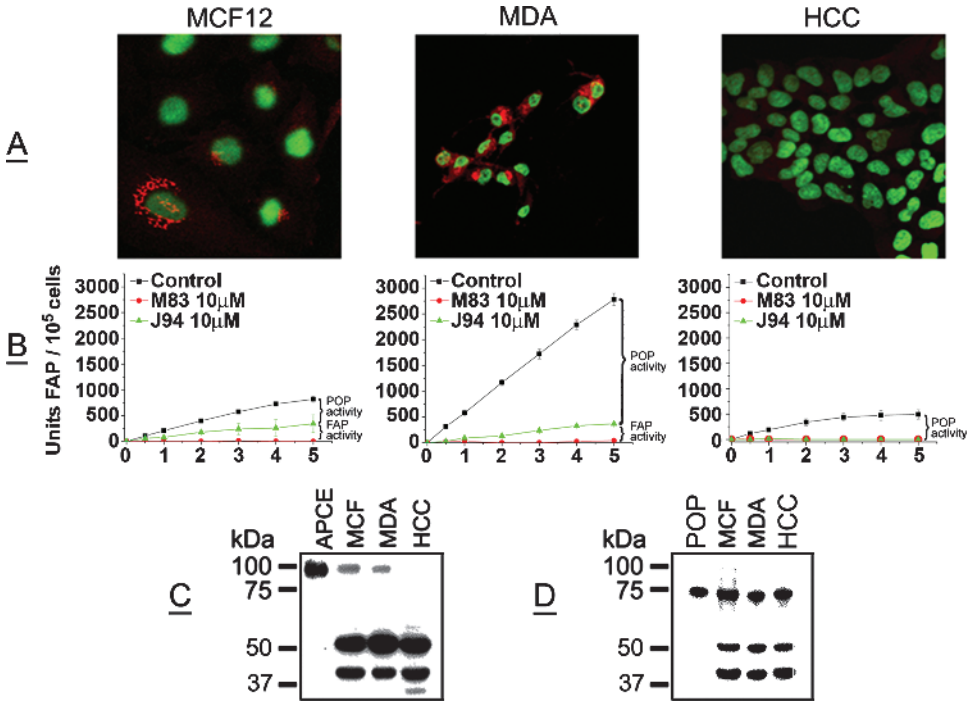

Figure 4. Characterization of FAP and POP in normal breast and breast cancer cells. (A) Confocal images of permeabilized MCF-12A, MDA, or HCCs grown on glass slides and labeled with mAb F19 to FAP followed by anti–mouse Alexa Fluor 568 (red) and DAPI (green). (B) FAP and POP activities on surfaces of normal and breast cancer cells grown on plastic wells as measured by cleavage of fluorescent substrate C95 and using the POP specific inhibitor J94 to separate the two activities. One FAP unit = Δfluorescence/min on cleavage of C95 by 1 nanogram of APCE. (C) Immunostains of cell lysates from normal and breast cancer cell cultures, using mAb 6D2 to FAP. APCE (1 ng) was used as a positive control, while intracellular contents of α-tubulin (50 kDa) and actin (43 kDa) were used for standardizing the amount of cell lysate protein applied in each lane. (D) Immunostains of cell lysates from normal and breast cancer cell cultures using goat anti-POP. POP (1 ng) was used as a positive control, while α-tubulin (50 kDa) and actin (43 kDa) served as load controls.

as the complexity of the tubule network increased. Interestingly, detectable FAP expression as reflected by proteolytic activity began ~3 to 4 hours after tubulogenesis (i.e., 8 hours from plating HMVECd) and continued to increase during the subsequent 18-hour period of growth. These findings suggest that POP may be critical to the initiation and propagation of vessel formation, with the timing of FAP expression synchronized with ECM invasion by the forming capillary-type tubules [19,22,38]. While FAP mRNA is upregulated coincident with capillary formation [39], the observation that proteolytically active FAP protein becomes easily detectable and increases progressively with tubulogenesis has not been reported previously. The temporal relationship of FAP production by ECs and the possibility that tissue-specific vascular FAP expression occurs could account for not always observing FAP in ECs [7,40].

As shown in Figure 7, when HMVECd and other selected cell types were stressed by removing hydrocortisone (hyc) from growth media, FAP became substantially overexpressed as detected by Western blots of cell lysates and corresponding increases in FAP proteolytic activity. Identification of the overexpressed protein as FAP was validated by amino acid sequence determination as shown in Figure 2. In Figure 7*A*, the greatest overexpression of FAP in response to omission of hyc in growth media was seen with MCF-12A. When hyc was restored to concentrations used for growth of normal cells in culture, overexpression of FAP by MCF-12A cells was totally reversed [Figure 7*B*, compare MCF-12A(–)hyc and MCF-12A(–)hyc/(+)hyc]. In contrast, removal of various growth factors (vascular endothelial growth factor, basic fibroblast growth factor, epidermal growth factor, and insulin-like growth factor) from growth media had no apparent effect, even after 42 days of culture.

Cancerous MSCs may be the best target in the search for a broadly applicable common denominator or "pan-tumor" approach for treating a large number of cancers [41,42]. We are aware of only one report showing FAP to be associated with MSC membranes as evidenced by immunoselection of MSCs from human cryopreserved bone marrow with a FAP mAb [43]. This prompted us to question whether MSCs, as the putative precursor to activated stromal fibroblasts [44], also expressed proteolytically active, membrane-bound FAP, and if so, how much relative to the activated fibroblast, and could it be readily inhibited? Confocal microscopy of permeabilized human mesenchymal cells in Figure 8*A* shows significant amounts of cytosolic and membrane-associated FAP, more or less similar to WI-38 fibroblasts. Western immunoblots of mesenchymal cell lysates (Figure 8*C*) confirmed a large amount of FAP present and did identify POP protein as well (Figure 8*E*). LC/MS/MS analysis of tryptic peptides from digestion of the ~100 kDa band in lysates of MSCs established the protein band as a subunit of homodimeric FAP (Figure 2). When mesenchymal membrane and cytosolic fractions were separated and subjected to Western blot analysis, it was clear that FAP is abundant on membranes and is in the cytosol to a lesser extent (Figure 8*D*). In contrast, the majority of POP is in the cytosol, with a lesser amount in the membrane fraction (Figure 8*F*). Mesenchymal cells and fibroblasts had about the same amount of FAP and POP activity, which might be expected given that mesenchymal cells are believed precursors of fibroblasts [44]. Assuming MSC and fibroblast membranes are impermeable

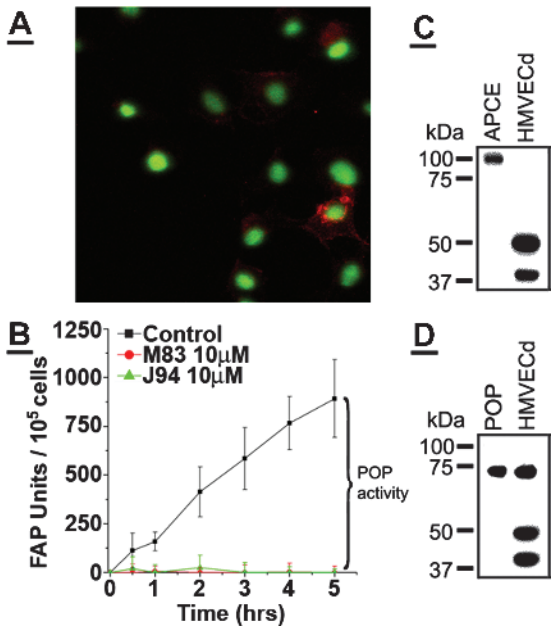

Figure 5. Characterization of FAP and POP in microvascular ECs. (A) Confocal images of permeabilized HMVECd grown on glass slides and labeled with mAb F19 to FAP followed by anti–mouse Alexa Fluor 568 (red) and DAPI (green). (B) FAP and POP activities of ECs grown on plastic wells measured by exactly the same methods described in Figures 1 and 4. (C) Immunostaining for FAP performed as described in Figures 1 and 4. (D) Immunostaining for POP performed as described in Figures 1 and 4.

to C95 substrate because of the latter's positive charge and lack of hydrophobicity, cytosolic FAP activity in live cells would not likely contribute to measurement of membrane activity.

Discussion

FAP continues to be a potential diagnostic or therapeutic target because it is 1) overexpressed by activated stromal fibroblasts in epithelial-derived human malignancies [11,12,22] and 2) absent in normal adult tissues and benign tumors [11,12,22]. Santos et al. [22] showed that targeted gene disruption or pharmacologic inhibition of FAP proteinase activity slowed or halted tumor growth in mouse models of endogenous lung cancer and human colon cancer. In both tumor scenarios, cancer cell proliferation decreased, collagen increased, and myofibroblast content and blood vessel density decreased. In those studies, however, the two inhibitors of FAP, e.g., Glu-boroPro or Val-boroPro, are also known to inhibit physiologically important DPPIV; both also inhibit POP; and both undergo cyclization, causing abbreviated *in vivo* survival that limits therapeutic potential.

Recently, Kraman et al. [45] reported that absence of FAP-expressing cells in mice allowed effective vaccination strategies for immunologic control of epithelial cell–derived cancer growth. In that study, no direct efforts were reported for determining whether FAP proteolytic activity was necessary for immunosuppressive effects within the tumor environment. In accord with both proteolytic and nonproteolytic roles for FAP in malignant growth, Huang et al. [46] recently suggested that FAP proteolytic function is important in ECM degradation but that other undefined properties (possibly immunologic) of FAP may promote tumor growth. While the function of FAP within malignancies remains poorly understood, most efforts to assess FAP as a therapeutic target have involved inhibiting its proteinase activity or using the latter to cleave oncologic drugs attached to peptides targeted to FAP [47]. Characterizing the proteolytic activity of membrane-inserted FAP has been unusually difficult, since a physiologic or pathologic substrate has not been definitively identified. We used the amino acid sequence surrounding the scissile bond of precursor α_2AP, a FAP substrate, to design stable and highly effective water-soluble inhibitors of APCE and FAP [28]. In the process, we discovered that a positively charged residue in P6 or P7 augmented substrate cleavage rate significantly [30,48], and this proved useful in developing our most effective FAP inhibitor, M83, and its analogue fluorescent substrate, C95 [28,30]. Both manifested high affinity and good specificity for FAP and, to a significant extent, for another prolyl-specific serine proteinase family member, namely, POP, which we, like others [34,49], found associated with selected normal and cancer cell lines.

We used the C95 FAP and POP substrate as well as the M83 inhibitor of both FAP and POP in conjunction with the highly specific J94 POP inhibitor, which had no effect on FAP, to quantitate and assign endopeptidase activity on cell membrane surfaces to its respective

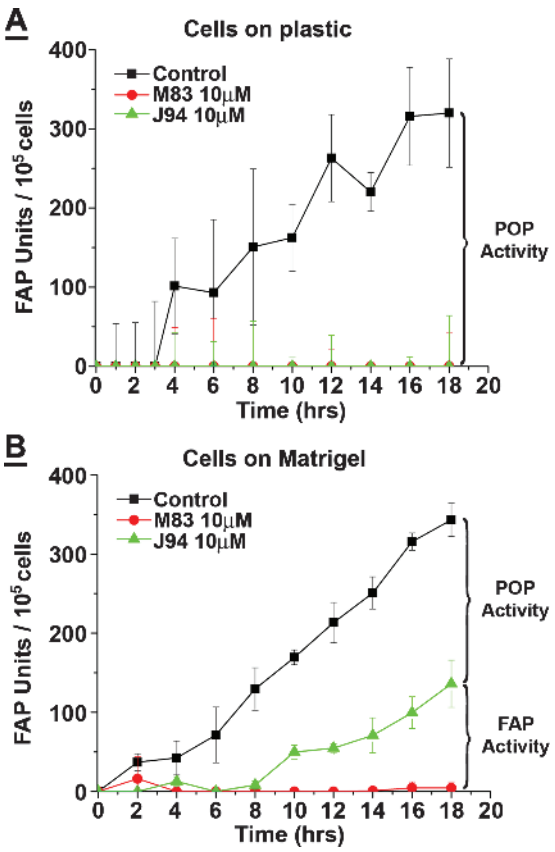

Figure 6. FAP and POP activities in microvascular ECs. (A) HMVECd were plated on plastic wells and allowed to settle for 1 hour, then inhibitors and substrates were added and fluorescence was measured over time for 18 hours. (B) HMVECd were plated on Matrigel, allowed to settle for 1 hour to initiate tubule formation, and assayed for FAP and POP activities as in A. Note that POP activity is detectable from the start in both assay conditions, but FAP activity only appears in the Matrigel assay at ~4 hours after tubule formation. Each point represents 15 readings over three separate experiments.

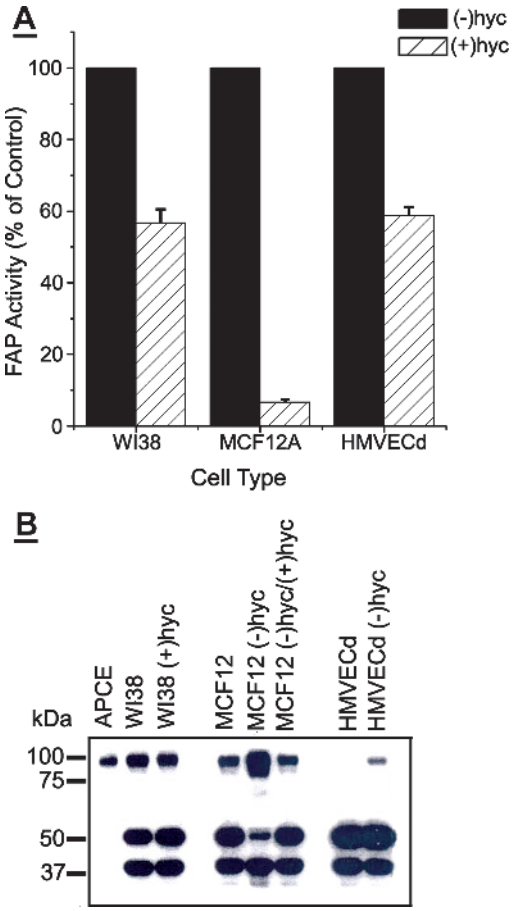

Figure 7. FAP activity and FAP protein levels in stressed cells. WI-38 fibroblasts, MCF-12A, and HMVECd were grown in the presence of hydrocortisone as customarily present in growth media or in its absence. Cells were grown from 7 to 14 days as specified before assessing FAP activity and FAP protein levels. (A) FAP activity was measured as previously described. FAP activity in the absence of hyc was set at 100%, with the level of FAP activity in the presence of hyc expressed as a relative percent. (B) Immunostains of cell lysates from fibroblast, normal breast, and EC cultures, using mAb 6D2 to FAP. APCE (1 ng) is used as a positive control; α-tubulin (50 kDa) and actin (43 kDa) were used to standardize cell lysate protein amounts applied to each electrophoretic lane. MCF-12A were grown in the absence of hyc, (−)hyc, for 7 days, followed by repletion of the normal media (+)hyc content, as supplied by Lonza, for 7 days, labeled (−)hyc/(+)hyc, before assessing both FAP activity and FAP protein concentration.

enzyme of origin. While POP cleaves selected peptides of less than about 30 residues, its biologic function remains unknown, as POP still lacks proven biologic substrate(s) that yield definitive physiologic effects [50]. Interestingly, however, POP recently has been proposed to have a significant role in angiogenesis by secondarily cleaving a derivative peptide from the ubiquitously tissue-distributed thymosin-β_4 to yield the tetrapeptide, acetyl-SDKP, a potent stimulator of angiogenesis [36].

Our results show that activated fibroblasts, i.e., those that are 1) rapidly dividing, 2) highly mobile, 3) contain α-smooth muscle actin (myofibroblasts), and 4) manifest enhanced ECM deposition, also have impressive amounts of FAP on their membranes with lesser amounts in the cytosol (Figure 1, *A* and *D*). Cancer-associated fibroblasts are activated fibroblasts that typically help form the stromal scaffolding of metastatic epithelial-derived tumor microenvironments. Assays of FAP or POP activity in the nonserum-containing media in which cells grew, or in buffer washes of those cell cultures, were always devoid of proteolytic activity, thereby supporting both enzymes as membrane-associated and in conformations that allow proteolytic activity to be easily and rapidly inhibited. Subtraction of the activity specifically inhibited by the J94 POP inhibitor provided an estimate of accessible FAP proteolytic activity. Assuming equivalent recoveries of FAP from different cell types, the amount of FAP protein and FAP proteinase activity in WI-38 fibroblasts exceeded that in any other cell, except for MSCs. Immunoreactive FAP protein was recovered from WI-38 fibroblasts and MDA, HMVECd deprived of hyc, or MSCs, and in each case gave essentially an identical amino acid sequence to that established for FAP [15]. Proteolytic assays confirmed that viral-transformed VA-13 human fibroblasts lacked both membrane-associated FAP and POP activities.

We demonstrated small amounts of FAP and POP activity and protein in normal breast cells, about the same amount of FAP activity in human metastatic breast cells; however, the latter cells contained about six times the amount of POP activity compared to normal breast cells. It seems reasonable that within hormonally induced cyclical tissue responses, an occasional normal breast parenchymal cell might be induced to express a small amount of FAP and POP. HCC1419 intraluminal primary breast cancer cells contained barely detectable FAP by immunostaining, and no assayable FAP proteinase activity; POP activity was greatly reduced, but POP protein, likely intracellular, was easily demonstrable. Within a growing HCC1419 tumor, however, this does not preclude the possibility that requisite stroma within the breast cancer malignancy may express FAP and POP. The basis of abundant POP activity found on MDA (Figure 4) remains obscure, despite such increases having been noted before in several other malignancies [26,34]. Larrinaga et al. [34] reported POP on cell membranes of various human cancers but usually in amounts not much different than those on corresponding normal cell types; however, cytosolic POP within cancer cells was regularly significantly increased beyond that in corresponding normal cells, which is in keeping with our finding for HCCs.

ECs (HMVECd) grown on plastic contained a rare FAP-positive cell by immunostaining but no detectable FAP protein by immunoblot analysis or proteinase assay; however, significant POP activity was expressed as confluence was achieved, perhaps related to its proposed role in capillary tube formation [36], but as expected, tube formation did not occur on plastic, and POP expression continued unabated. FAP was not expressed during the 72-hour growth period (Figure 5*B*). In contrast, when grown on Matrigel, by about 4 hours the HMVECd cells began to align progressively in tubular structures as POP expression continued to increase (Figure 6*B*). Interestingly, FAP activity became demonstrable shortly after tube-like capillaries began forming (Figure 6*B*), and by ~18 hours, well-defined capillary-like networks dominated. These results prompted the speculation that expression of proteolytically active FAP might be synchronized with capillary growth to foster invasiveness of developing microvasculature into the ECM [38]. Aimes et al. [39] noted endothelial expression of mRNA transcripts of several serine proteinases (including FAP) in association with the nature of the culture substratum and progression of angiogenesis. Our finding of increasing FAP proteolytic activity expands their report by demonstrating that increased FAP mRNA is actually translated during tubule formation. Interestingly, Cavallo-Medved et al. [51] noted that concentrations of other selected proteinases

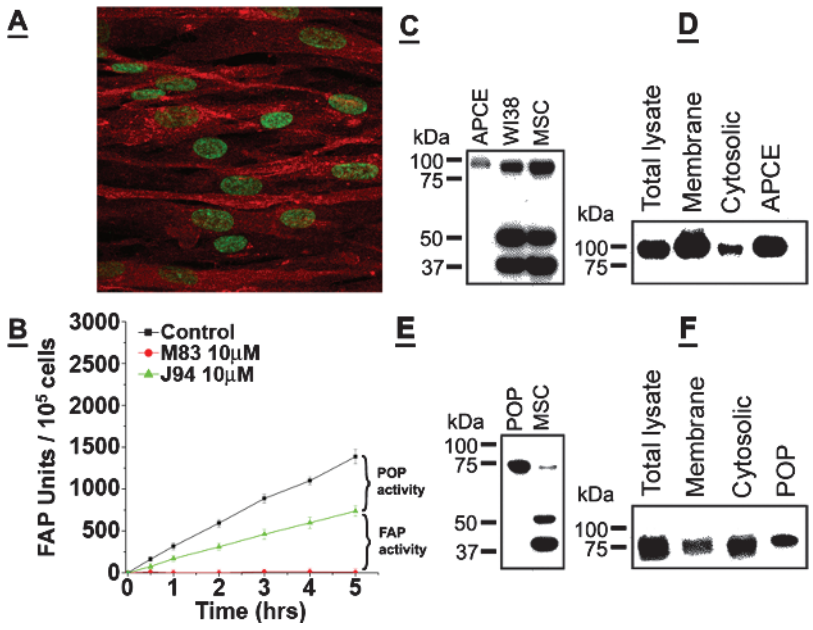

Figure 8. Identification and characterization of FAP and POP in human MSCs. (A) Confocal image of permeabilized MSCs was grown on glass slides and labeled with mAb F19 to FAP followed by anti–mouse Alexa Fluor 568 (red) and DAPI (green). (B) FAP and POP activities found on MSC surfaces grown in plastic wells were measured exactly the same as described in Figures 1 and 4. (C) Immunostaining for FAP was performed as described in Figures 1 and 4. (D) Immunostaining for FAP in cell lysates and membrane and cytosolic fractions of MSCs was performed as described in Figure 1. (E) Immunostaining for POP was performed as described in Figures 1 and 4. (F) Immunostaining for POP in cell lysates and membrane and cytosolic fractions of MSCs was performed as described in Figure 1.

appeared to increase in conjunction with advancing gelatinolytic activity during *in vitro* tube formation. A few other studies have also suggested that ECs within the developing microvasculature of malignant tissues do indeed express FAP [7,40,52].

As shown by immunoblot analysis and activity assays (Figure 7), stress caused by removal of hyc from growth media stimulated both HMVECd and MCF-12A to overexpress FAP as identified by amino acid sequence (Figure 2). Notably, replenishment of hyc returned FAP expression to levels before hyc removal (Figure 7). Similarly, WI-38 activated fibroblasts, which are ordinarily grown in the absence of hyc, showed a detectable decrease in FAP expression when hyc was added. Analogous effects with hyc have been noted before, e.g., decreased hyc in normal human epithelial cells was associated with increases of urokinase and tissue plasminogen activator [53]. These data suggest that cells are capable of altering expression of FAP if they should become stressed as might occur in a rapidly expanding metastatic tumor microenvironment.

MSCs give rise to progenitor adipocytes, bone cells, and myocytes and are also considered precursive to fibroblasts [44,54]. Conceivably, activated MSCs could arise as a consequence of epithelial-mesenchymal cell transformation [55] and represent the progenitor cell of the "tumor niche." Some propose, however, that MSCs originate from the bone marrow, move into a selected tissue, and undergo malignant transformation to produce cancer cells characteristic of that tissue [41,56]. Others consider that a precursive cancer stem cell might derive from a dormant multipotent cell unique to a specific tissue. The amounts of FAP we observed for MSCs by confocal microscopy and Western blot analysis (Figure 8) were more than for WI-38 activated fibroblasts (Figure 1). The multiple roles of mesenchymal cells suggest they may exist in an activated state more frequently than do fibroblasts. This may be particularly true with respect to mesenchymal cell involvement in malignancies and account for the large amount of FAP we observed in these cells.

Our results allow the following conclusions: 1) FAP is uniquely expressed on the membranes of cells critical to tumor niche formation in primary tumors or metastases, namely, cancer-associated fibroblasts, MSCs, selected cancer cells, and ECs as the latter participate in angiogenesis; POP in general is overexpressed in cells of the tumor niche. 2) In their membrane-bound form, each enzyme is proteolytically active and easily accessible for efficient inhibition by a new soluble, high-affinity, selective pseudo-peptide inhibitor that meets structural requirements for the respective enzyme's substrate-binding region. 3) ECs readily express POP as they grow, and in addition, membrane-inserted proteolytically active FAP is synthesized and expressed as tubulogenesis occurs [39,52]. 4) FAP and POP hold promise as therapeutic targets for a large number of cancers and the two new inhibitors, M83 and J94, may be useful for determining if inhibition of one or both proteolytic activities will deter or abolish growth of commonly encountered epithelial-derived cancers and their metastatic foci. Results reported here now point toward further studies of the inhibitors' potential therapeutic effects on selected epithelial-derived human tumor xenograft models in immunocompromised mice.

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