Fibroblast Activation Protein: Differential Expression and Serine Protease Activity in Reactive Stromal Fibroblasts of Melanocytic Skin Tumors

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Growth and metastasis of solid neoplasms require the recruitment of a supporting tumor stroma. A highly consistent trait of tumor stromal fibroblasts in most epithelial cancers is the induction of fibroblast activation protein (FAP), a member of the serine protease family. Recently it was demonstrated that FAP has both dipeptidyl peptidase and collagenolytic activity capable of degrading gelatin and type I collagen. In this study, we describe the expression and enzyme activity of FAP in benign and malignant melanocytic skin tumors. FAP-positive fibroblasts were detected immunohistochemically in the reactive stroma of all melanocytic nevi tested. In primary and metastatic melanomas an upregulation of FAP expression in the reactive mesenchyme could be observed. Whereas 30% of the nevi revealed additional FAP expression on subsets of melanocytic cells, melanoma cells from primary and metastatic melanomas were FAP negative. This may indicate a possible role for FAP in the control of tumor cell growth and proliferation during melanoma carcinogenesis. Consistent with this *in vivo* expression pattern FAP enzyme activity could be detected by a specific immunocapture assay in extracts of melanocytic nevi and melanoma metastases, whereas no significant activity was detectable in normal adult skin. Strong protein expression of FAP was observed in patterned structures restricted to a subset of the melanoma metastases. Our findings that these FAP-positive structures showed no overlap with endothelial cell surface markers, nor with various melanoma antigens, suggest that FAP is a marker for specific stromal-cell-derived patterns in cutaneous melanoma metastases.
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The invasive growth of solid tumors requires a number of cellular and molecular changes in the supporting stroma, including formation of lymphoid and phagocytic infiltrates, release of peptide mediators and proteolytic enzymes, and the production of an altered extracellular matrix. The complex interplay between the different cell types involved, including the malignant cells themselves, the endothelium, and tumor stroma fibroblasts, is increasingly understood (Ruiter *et al*, 2002). One key aspect of tumor stroma formation in solid neoplasms is the production of new blood vessels (angiogenesis) to provide an enhanced tumor blood supply (Hanahan and Folkman, 1996). In addition, the supporting stroma of most types of epithelial cancers contains an abundance of specialized fibroblasts, referred to as reactive tumor stromal fibroblasts. Whereas tumor stromal fibroblasts are not transformed, they differ from resting fibroblasts in normal adult tissues by a characteristic gene expression pattern (Scanlan *et al*, 1994). A highly consistent and specific molecular trait of tumor stromal fibroblasts in several types of epithelial cancers is the induction of fibroblast activation protein (FAP), a member of the serine protease family (Garin-Chesa *et al*, 1990). FAP is an M_r 95,000 cell surface protein, as detected by the monoclonal antibody F19 (Garin-Chesa *et al*, 1990). As a type II membrane-bound glycoprotein, FAP shows close kinship to the heterodimeric partner dipeptidyl peptidase IV (DPP-IV/CD26), which is widely expressed in normal tissues with roles in T cell costimulation, chemokine biology, type II diabetes, and tumorigenesis (Scanlan *et al*, 1994; Wesley *et al*, 1999). In contrast, immunohistologic studies have demonstrated a highly selective FAP expression in stromal fibroblasts of over 90% of common human epithelial cancers (Garin-Chesa *et al*, 1990), in the granulation tissue of healing wounds, and in a large proportion of bone and soft tissue sarcomas (Rettig *et al*, 1988). FAP is not expressed in epithelial tumor cells, and is also not detectable in stromal fibroblasts of benign epithelial tumors, or normal adult tissues. The restricted distribution of FAP in normal tissues and benign epithelial tumors in contrast to its abundant expression in the stroma of a large proportion of epithelial cancers has provided the rationale for immunotherapy strategies aimed at the tumor stroma rather than surface

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Abbreviations: DPP-IV, dipeptidyl peptidase IV; FAP, fibroblast activation protein.

molecules of malignant cells (Rettig *et al*, 1993). Recently it was shown that FAP has both dipeptidyl peptidase and a collagenolytic activity capable of degrading gelatin and type I collagen (Park *et al*, 1999). In this study it was concluded that (i) FAP belongs to the serine protease family rather than the matrix metalloprotease family and (ii) cleavage of gelatin and native collagen I places FAP into the group of enzymes possibly involved in tumor tissue remodeling by affecting extracellular matrix degradation and/or growth factor activation.

Malignant melanoma is among the most aggressive tumors in humans with minimal progress in nonsurgical treatment of advanced disease (Byers and Bhawan, 1998; McMasters *et al*, 1999). In contrast to the more frequently occurring basal cell and squamous cell carcinoma malignant melanoma is the most common fatal malignancy among young adult Caucasians, and its incidence and mortality continue to increase at an alarming rate (Halachmi and Gilchrest, 2001). Although substantial progress has been made in understanding the genetic changes and molecular processes resulting in transformation of benign melanocytic nevi to malignant melanomas (Chin *et al*, 1998; Ruiter *et al*, 2002), fundamental mechanisms of tumor cell invasion and metastasis of primary melanomas remain unknown to date. Expression profiling studies using microarrays have revealed that growth factors, cell adhesion molecules, proteases, and molecules involved in intracellular signal transduction are differentially expressed in metastatic melanoma cells, suggesting important roles for melanoma progression (Bittner *et al*, 2000; Clark *et al*, 2000). FAP may play an important role during melanoma progression, as cultured normal melanocytes express FAP/DPP-IV, whereas malignant melanoma cell lines are FAP/DPP-IV negative or express only FAP in culture (Rettig *et al*, 1993). Furthermore, in an *in vitro* model of melanoma pathogenesis, v-Ha-*ras* transformation of normal cultured melanocytes leads to partial growth factor independence and – together with a fully transformed phenotype (Houghton *et al*, 1988; Albino *et al*, 1992) – to successive stages of FAP loss (Rettig *et al*, 1993). Although most molecular studies in general originally focused on melanoma cells, it has become increasingly evident that stromal cells, in particular fibroblasts, play key roles in processes of melanoma growth and metastasis, including proliferation, matrix degradation, and migration (Liotta *et al*, 1991; Fidler, 1995; Ruiter *et al*, 2002). Nothing so far is known about the *in vivo* expression pattern of FAP in melanocytic skin lesions – both in the stroma as well as on tumor cells – during melanoma initiation and progression.

Therefore, this study was designed to determine FAP expression in a series of benign and malignant melanocytic lesions *in vivo* and to link the *in vivo* expression pattern with the known biochemical function that may help to explain its role in tumorigenesis and stroma remodeling events during transformation and tumor progression.

MATERIALS AND METHODS

Tissues This study was approved by vote no. 117/2001 of the Ethical Committee of the University of Ulm. After individual informed written consent was given according to the guidelines, tissues were obtained from surgical specimens, embedded in OCT compound, quick-frozen by the isopentane/liquid nitrogen method, and stored at –80°C. Cryostat sections (5 μm) were cut, air-dried, and fixed in cold acetone. The avidin–biotin complex immunoperoxidase staining procedure was carried out as described below. Tumor diagnoses were established through routine pathologic evaluation of paraffin-embedded tissues in the Departments of Dermatology at the University of Ulm and Armed Forces Hospital of Ulm. For all immunohistochemical assays, parallel sections were stained with hematoxylin–eosin for additional histologic evaluation.

Immunohistochemistry The avidin-biotin complex immunoperoxidase method was carried out as previously described (Garin-Chesa *et al*, 1987; 1988). Briefly, 5 μm thick sections were cut, mounted on poly(L-lysin)-coated slides, air-dried, and fixed in cold acetone (4°C, for 10 min). Sections were treated with 0.3% H_2O_2 for 3 min to block endogenous peroxidase followed by blocking with normal horse serum for 30 min at room temperature. Slides were incubated at 4°C with monoclonal antibodies F19 (raised against Fibroblast Activation Protein, as described by Rettig *et al*, 1988) and H572 (anti-Endoglyx-1, as described by Sanz-Moncasi *et al*, 1994), hybridoma culture supernatants, diluted 1:20; mouse antihuman CD31/PECAM-1 (Dako, Glostrup, Denmark, diluted 1:100); mouse anti-S100 protein (Sigma, St. Louis, MO, diluted 1:5000); mouse antihuman CD1a (Pharmingen, San Diego, CA, diluted 1:500); mouse anti-gp100 (HMB45, Dako, diluted 1:500); or unrelated mouse IgG-negative control antibodies (1–5 μg per ml) for 12–18 h. Sections were washed and incubated with biotinylated horse antimouse IgG (1:100; Vector Laboratories, Burlingame, CA) for 30 min at room temperature, followed by avidin––biotin–horseradish peroxidase complex (1:100 dilution at a 1:1 ratio). The final reaction product was visualized with the chromogen 3,3′-diaminobencidine. Sections were counterstained with Harris's hematoxylin.

Indirect immunofluorescence and confocal microscopy Coexpression of FAP, Endoglyx-1, and gp100 was studied in a subset of the melanoma specimens. For this purpose, the sections were incubated sequentially with the corresponding primary antibodies followed by differentially labeled secondary antibodies, using an indirect immunofluorescence method. Goat antimouse IgG conjugated with Alexa 488 (Molecular Probes, Eugene, OR) was used for FAP expression (green fluorescence) followed by a goat antimouse IgG Alexa 546 (Molecular Probes) conjugated antibody for Endoglyx-1 expression (red fluorescence). FAP expression and gp100 colocalization was studied by sequential incubation with F19/Cy2 conjugated goat antimouse followed by an anti-gp100/Cy3 conjugated goat antimouse antibody (Jackson, West Grove, PA, Dianova, Hamburg, Germany). Sections stained by these double immunofluorescence procedures were examined and photographed with a Leica TCS spectral confocal microscope.

Preparation of membrane extracts and dipeptidyl peptidase assays A quantitative assay for dipeptidyl peptidase activity was performed using Ala-Pro-AFC as the substrate, as described previously (Park *et al*, 1999). In brief, frozen samples of tumor tissues or normal skin (stored at –80°C) were thawed on ice, diced with a scalpel, snap frozen in liquid nitrogen, and pulverized with a mortar and pestle. Extraction buffer (150 mM NaCl, 50 mM Tris–HCl buffer, pH 7.4, 10 mM $MgCl_2$, 1% Triton X-114) was added, and the resulting slurry was homogenized with an Ultorax homogenizer for about 1 min and subsequently placed on ice for 20 min. Extracts were cleared by centrifugation ($4000 \times g$) for 10 min at 4°C and phase-partitioned at 37°C for 10–20 min, followed by centrifugation ($4000 \times g$, 20 min at room temperature), without braking. The detergent phase was diluted with 150 mM NaCl, 50 mM Tris–HCl buffer, pH 7.5, 5 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.75% Empigen BB, and separated on concanavalin A Sepharose. Concanavalin-A-bound fractions were eluted with 1 M methyl-α-D-mannopyranoside, 150 mM NaCl, 50 mM Tris–HCl buffer, pH 7.4, and 0.1% Empigen BB. Membrane extracts were tested for FAP activity via an immunocapture step. Enzyme-linked immunosorbent assay (ELISA) plates (96 well, Costar, Corning, NY) were coated overnight at 4°C with 1 μg per ml chimeric F19 antibody or control antibody in phosphate-buffered saline (PBS). Wells were then rinsed with wash buffer (PBS, 0.1% Tween 20), and excess binding sites were blocked with blocking buffer (5% bovine serum albumin in PBS) for 1 h at room temperature. Blocking buffer was removed; membrane extracts were added and incubated for 1 h at room temperature. The unbound material was removed, wells were washed thrice with wash buffer, and dipeptidyl peptidase activity was assayed using 100 μl of Ala-Pro-AFC (0.5 mM Ala-Pro-AFC in reaction buffer) for 1 h at 37°C. Background fluorescence (as measured using control antibody) was subtracted from each value. Release of free AFC was measured in a Cytofluor fluorimeter (PerSeptive Biosystems, Applied Biosystems, Foster City, CA) using the 395 nm excitation/530 nm emission filter set.

RESULTS

FAP is induced in activated stromal fibroblasts of benign melanocytic nevi In order to determine FAP protein expression in melanocytic nevi we carried out an immunohistochemical analysis of FAP distribution in nevi using monoclonal antibody F19. Immunostaining demonstrated positive staining of stromal fibroblasts in all nevi ($n = 19$), regardless of the histopathologic subtype (**Fig 1**, **Table I**). These F19-positive stromal cells show a mesenchymal phenotype and express various commonly used mesenchymal markers, such

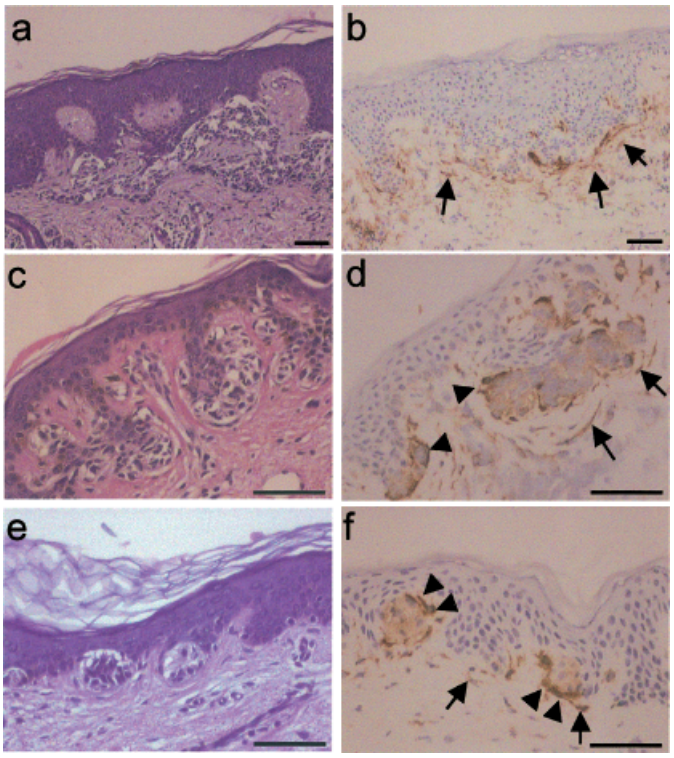

Figure 1. Immunohistochemical detection of FAP in melanocytic nevi with monoclonal antibody F19. Melanocytic nevi (*A–F*) were stained either with hematoxylin-eosin (*A*, *C*, *E*), or with monoclonal antibody F19 to detect FAP expression (*B*, *D*, *F*). FAP immunoreactivity was seen in the reactive stromal fibroblasts surrounding the nevi (*arrows*; *B*, *D*, *F*), but also on subsets of melanocytic cells at the dermo-epidermal junction (*arrowheads*; *D*, *F*) in compound nevi (*C*, *E*). *Scale bar*: 50 μm.

as vimentin and α-smooth muscle actin (data not shown), consistent with fibroblasts and myofibroblasts. In most cases, normal skin in distant and close vicinity to the melanocytic lesion was also available for analysis. Interestingly, stromal cells in normal skin adjacent to the nevi were F19 negative, whereas F19 expression was already detectable in areas of inflammation (data not shown) and in reactive mesenchyme directly surrounding the lesions (**Fig 1*B*, *D*, *F***). The induction of FAP in activated stromal fibroblasts of benign melanocytic lesions is in contrast to the findings in benign and premalignant epithelial tumors, which generally lack FAP-positive stromal cells (Garin-Chesa *et al*, 1990).

FAP is expressed on melanocytic cells in a subset of melanocytic nevi In a subset of the melanocytic nevi FAP was not only detected on stromal fibroblasts but also on subsets of melanocytic cells present at the dermo-epidermal junction (**Fig 1*D*, *F***). In each case, the histopathologic diagnosis was confirmed and different cell types were identified by thorough morphologic evaluation of hematoxylin–eosin stained sections of the lesions (**Fig 1*A*, *C*, *E***). In addition, we observed expression of HMB-45 in some of the FAP-expressing nevus cells, and expression of S100 protein in most of the FAP-positive nevus cells in adjacent sections, respectively (see *Materials and Methods*, data not shown). Of all cases studied ($n = 19$), we found F19-positive melanocytic cells in approximately one-third of all nevi ($n = 6$), regardless of the histopathologic subtype (see **Table I**). This is again in contrast to the findings in benign and malignant epithelial tumors that generally lack FAP expression on the surface of epithelial tumor cells (Rettig *et al*, 1993).

FAP expression is induced in stromal fibroblasts of malignant melanomas and melanoma metastases In order to determine the pattern of FAP expression in activated stromal

Table I. Summary of FAPα expression in melanocytic skin tumors

		F19 expression			
		Stroma			
Tumor type	*n*	++	+	−	Melanocytic/melanoma cells
Melanocytic nevus					
Compound	13		13		4
Congenital	3		3		1
Dermal	1		1		–
Dysplastic	2		2		1
In situ/primary melanoma					
In-situ MM	1		1		–
LM	2		2		–
SSM	2	2			–
NMM	1	1			–
ALM	1	1			–
LMM	1	1			–
Metastatic melanoma	12[a]	12			

Acetone-fixed frozen sections were tested by the avidin–biotin complex immunoperoxidase procedure and results are indicated as follows: ++, +, abundant and moderate numbers of F19-positive stromal fibroblasts, respectively; −, antigen-negative; MM, malignant melanoma; LM, lentigo maligna; SSM, superficial spreading melanoma; NMM, nodular malignant melanoma; ALM, acrolentiginous melanoma; LMM, lentigo maligna melanoma.
[a]12 samples obtained from seven different patients.

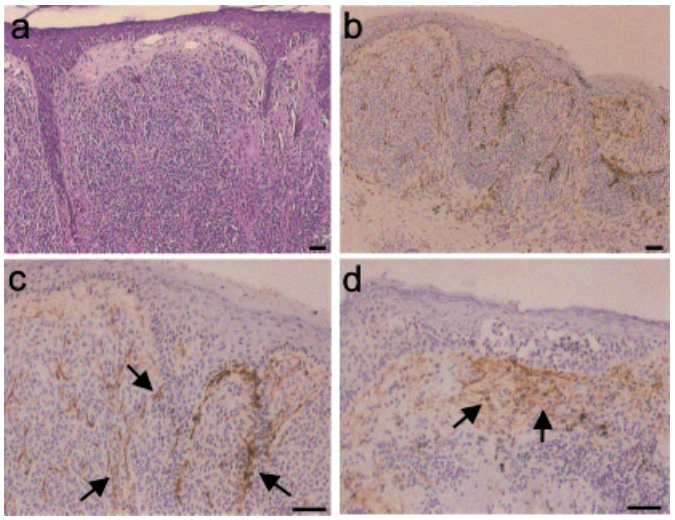

Figure 2. Immunohistochemical detection of FAP in primary melanomas with monoclonal antibody F19. Primary melanomas (*A–D*) were stained either with hematoxylin-eosin (*A*), or with monoclonal antibody F19 to detect FAP expression (*B–D*). FAP immunoreactivity was detected in stromal fibroblasts around the melanoma cell clusters (*arrows*). *Scale bar*: 50 μm.

fibroblasts during transformation of benign melanocytic nevi to malignant melanomas, a series of primary cutaneous melanomas ($n = 6$), melanoma metastases ($n = 12$), and two cases of lentigo maligna were investigated. The primary melanomas comprised the following histopathologic subtypes: superficial spreading ($n = 2$), nodular ($n = 1$), acrolentiginous ($n = 1$), and lentigo maligna melanoma ($n = 1$) (**Table I**). In addition, one case of an *in situ* melanoma was stained for FAP expression. FAP expression was seen in activated stromal fibroblasts in all cases (**Table I**; **Figs 2, 3**). There was no significant difference in the FAP expression between primary and metastatic melanomas. In all

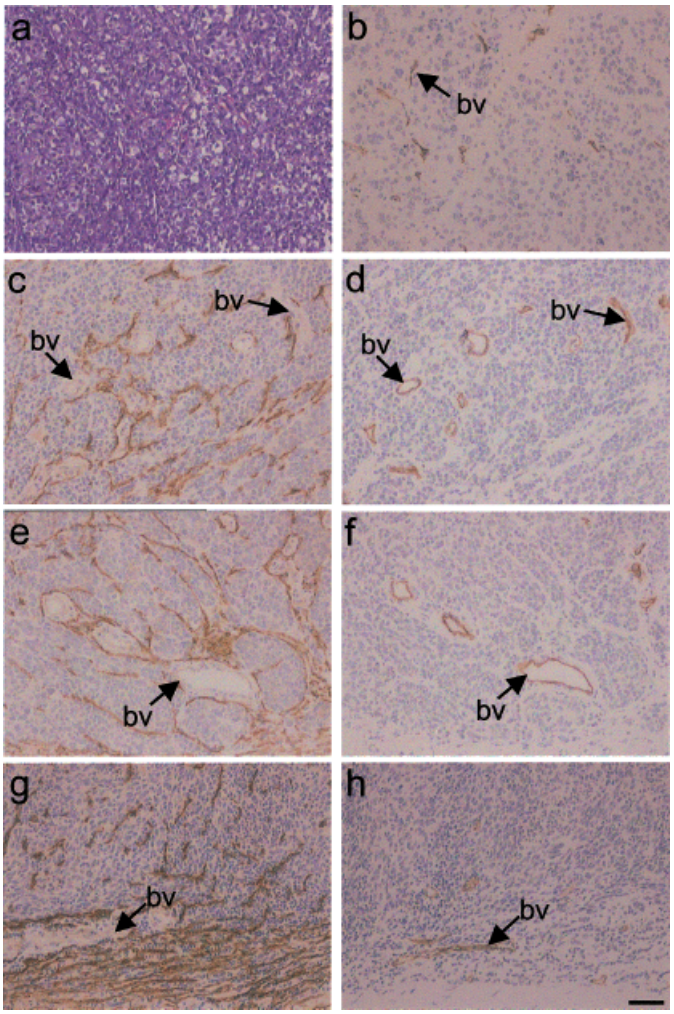

Figure 3. Immunohistochemical detection of FAP and Endoglyx-1 in metastatic melanomas. Melanoma metastases (*A–H*) were stained with hematoxylin-eosin (*A*), with monoclonal antibody F19 (*C, E, G*), or with monoclonal antibody H572 to detect the endothelial-specific Endoglyx-1 protein (*B, D, F, H*). Immunostainings of adjacent serial sections (*C/D, E/F, G/H*) demonstrate that conventional tumor blood vessels (bv) are Endoglyx-1 positive (*D, F, H*) and FAP negative (*C, E, G*). Note the networks of FAP-positive (Endoglyx-1-negative) loops and channels observed in a subset of metastatic melanomas (*C, E, G*). *Scale bar*: 50 μm.

cases, hematoxylin–eosin staining was performed for histolopathologic evaluation (**Figs 2*A*–3*A***). The upregulation of FAP in stromal fibroblasts of melanocytic malignancies resembles the findings in most epithelial cancers and supports the hypothesis that this protease plays an important role in the proliferative program of fibroblastic cells. In contrast to the melanocytic nevi, none of the malignant melanocytic tumors tested express FAP on the melanoma cells themselves (**Figs 2*B–D*–3*C*, *E*, *G***).

FAP expression in stromal patterns of cutaneous melanoma metastases In hematoxylin–eosin stainings of cutaneous melanoma metastases, generally only a limited number of blood vessels (resulting from tumor angiogenesis) is found (**Fig 3*A***). Conventional blood vessels are lined by endothelial cells, which can be defined by a panel of monoclonal antibodies against endothelial cell surface proteins. Besides the commonly used endothelial marker CD31, the highly endothelial lineage-specific cell surface glycoprotein Endoglyx-1 (Sanz-Moncasi *et al*, 1994; Christian *et al*, 2001) was used as a marker for identification of endothelial cells in cutaneous melanoma metastases by immunohistochemical staining with monoclonal antibody H572 against Endoglyx-1 (**Fig 3*B***). F19 and H572 immunostainings of corresponding sections of metastatic melanomas demonstrate clearly that tumor blood vessels are FAP negative (**Fig 3*C*, *E*, *G***) but show positive immunoreactivity for Endoglyx-1 (**Fig 3*B*, *D*, *F*, *H***). In each case, immunostaining for an additional endothelial cell surface marker, CD31, was carried out and showed immunoreactivity of the Endoglyx-1-positive structures (data not shown). Surprisingly, we observed dense networks of interconnected FAP-positive structures (loops, septa, and channels) in a subset ($n = 6$) of the F19-stained metastatic melanomas ($n = 12$; **Fig 3*C*, *E*, G**) that do not show Endoglyx-1 immunoreactivity in adjacent serial sections (**Fig 3*D*, *F*, *H***). As the characteristics of these structures were unclear, we next focused on the further analysis of the FAP-expressing cells constituting these patterned structures. For this purpose, double immunofluorescence experiments with anti-FAP and anti-Endoglyx-1 were performed in sections of metastatic melanomas and the results were evaluated by confocal microscopy. As illustrated in **Fig 4*A–C***, there was clearly no overlap between the F19-positive structures (green fluorescence) and the H572-positive tumor blood vessels (red fluorescence). In some sections, however, the network of F19-positive structures appears to have close contact to the vascular endothelium (**Fig 4*A***). As the FAP-expressing cells lining the observed structures could not be identified as vascular endothelial cells, we asked whether these FAP-positive structures may be related to the recently described and possibly tumor-cell-generated vascular channels found in aggressive uvea or metastatic human melanomas (Maniotis *et al*, 1999). Immunohistochemistry of adjacent serial sections from the samples shown in **Figs 3** and **4** were then stained for expression of melanoma antigens. Colocalization of FAP and the melanoma marker gp100 was studied by sequential incubation of metastatic melanoma sections with F19 (green fluorescence) followed by anti-gp100/HMB45 (red fluorescence). Confocal microscopy showed no overlap between the FAP-positive cells constituting interconnected channel- or loop-like patterns (**Fig 4*D–F***; green fluorescence) and the gp100-positive melanoma cells (**Fig 4*D–F***; red fluorescence) that are clustered around these septa. Intriguingly, cutaneous metastases excised 3–5 mo later from the same patients who were tested positive for this stromal feature ($n = 3$) revealed the same FAP-positive structures that were consistently negative for endothelial (CD31, Endoglyx-1) and melanoma (gp100, S100) cell markers (data not shown). Based on these expression data it is tempting to speculate that FAP may constitute a previously unrecognized marker for specific stromal patterns found in a subset of metastatic melanomas.

Enzymatic FAP activity in melanocytic nevi and melanoma metastases Given the fact that FAP protein expression is detectable in the stroma of melanocytic nevi and following transformation to malignant melanomas we next asked if FAP is present as an active cell-surface-bound protease in the stroma of melanocytic tumors. Recently, suitable test substrates for FAP enzyme function, such as Ala-Pro-AFC, have been identified (Park *et al*, 1999), and by establishing a FAP-specific enzyme immunocapture assay (Park *et al*, 1999) it has become feasible to monitor FAP activity directly in samples of human tumor tissues. Equal amounts of specimens of normal skin, melanocytic nevi, and melanoma metastases were extracted and subjected to enzyme immunocapture on monoclonal antibody F19-coated ELISA plates. Bound dipeptidyl peptidase activity measured with Ala-Pro-AFC as substrate showed a distinctive pattern, as indicated in **Fig 5**. FAP activity could be detected in extracts of all four melanocytic nevi and appears to be upregulated in melanoma metastases ($n = 6$), whereas little or no activity was detectable in biopsy specimens of normal skin ($n = 5$), correlating well with results from immunohistochemical staining. We conclude from these results that FAP is expressed as an active protease in benign melanocytic skin tumors and its increase in enzyme activity correlates with increased protein expression following transformation to malignant melanomas.

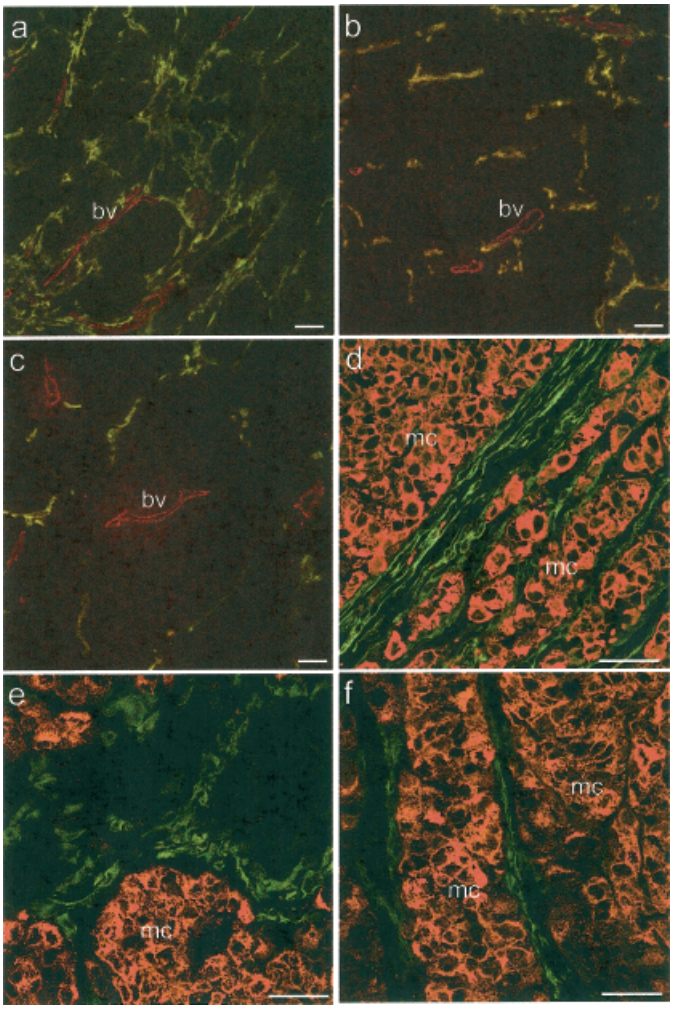

Figure 4. FAP expression on patterned structures does not overlap with endothelial and melanoma cell markers. Sections from metastatic melanoma lesions were examined under a confocal microscope for colocalization of FAP with endothelial markers. Double stainings were carried out with monoclonal antibody F19 detected by Alexa-488-conjugated secondary antibody (green fluorescence, *A–C*) and with monoclonal antibody H572 detected by Alexa-546-conjugated secondary antibody (red fluorescence, *A–C*) staining endothelial cells of blood vessels (bv). Monoclonal antibody F19 detected with Cy2-conjugated secondary antibody (green fluorescence, *D–F*) and anti-gp100/HMB45 detected with Cy3-conjugated antibody (red fluorescence, *D–F*) were used for colocalization of FAP and the melanoma marker gp100 staining melanoma cells (mc). Color overlay analysis demonstrates no colocalization of FAP with Endoglyx-1 (*A–C*) or with gp100 (*D–F*). *Scale bar*: 50 μm.

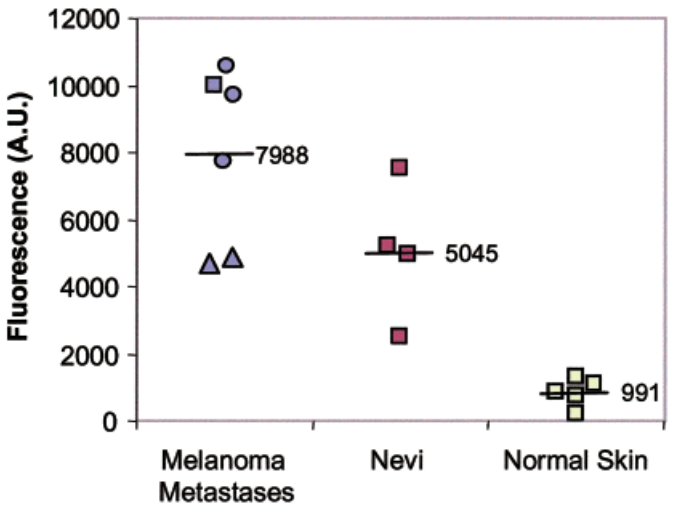

Figure 5. Dipeptidyl peptidase activity of FAP measured in extracts of human melanoma metastases (blue symbols), melanocytic nevi (red symbols), and matched normal skin (open symbols). Melanoma samples included three specimens from one individual (*circles*) and two specimens from another patient (*triangles*); all other samples in this figure (*squares*) were from different patients. FAP activity was measured using concanavalin-A-enriched membrane fractions, followed by binding to F19-coated plates and incubation with Ala-Pro-AFC as substrate. The bars and respective numbers represent the mean of each category. The differences between the means for melanoma metastases and melanocytic nevi *versus* normal skin, respectively, are significant at $p < 10^{-4}$ by Student's *t* test.

DISCUSSION

With these studies we have for the first time systematically determined the expression pattern and enzymatic activity of FAP in a series of melanocytic lesions ranging from benign melanocytic nevi to metastatic melanoma. FAP protein is expressed in stromal fibroblasts of all melanocytic tumors, including benign, premalignant, and malignant lesions. The observed upregulation of FAP expression and enzymatic activity during malignant transformation from benign melanocytic nevi to malignant melanoma is consistent with its upregulation in more than 90% of all investigated malignant epithelial tumors (including breast, colorectal, ovarian, lung, and pancreatic carcinomas; Garin-Chesa *et al*, 1990; Scanlan *et al*, 1994). In contrast to the findings in benign epithelial tumors, however, in which no or very little expression of FAP was observed on stromal fibroblasts (Garin-Chesa *et al*, 1990), we detected considerable expression and enzymatic activity of FAP in stromal fibroblasts of benign melanocytic skin tumors. The presence of FAP expression and activity in stromal fibroblasts of melanocytic nevi either suggests that FAP expression does not perfectly correlate with the activation state of stromal fibroblasts in melanocytic tumors, or emphasizes the fact that melanocytic nevi can be considered as precursor lesions for melanoma development (Clark *et al*, 1984; Elder *et al*, 1989), characterized by an – at least partially – constitutively active tumor stroma.

Little is known about the molecular changes that accompany activation of stromal fibroblasts during tumor progression, and gene expression profiling studies would be highly informative to address this question. The molecular response of resting fibroblasts to serum *in vitro* has been described using microarrays (Iyer *et al*, 1999), but provides only a first approximation of possible gene expression changes associated with the activation of stromal fibroblasts during tumorigenesis. FAP appears to constitute an ideal marker for expression profiling of stromal fibroblasts at different stages of melanoma progression, as a first step to identify regulatory pathways in these cells linked to tumorigenesis. FAP is detectable as an active serine protease in the reactive tumor stroma of melanocytic skin tumors, showing a close correlation between the presence of immunohistochemically detectable FAP and its dipeptidyl peptidase activity. The identification of key substrates of FAP may provide an additional entry point in understanding the pathways that control the activation of stromal fibroblasts during tumorigenesis.

FAP in addition may play a distinct role in the regulation of growth and transformation programs of melanocytic cells. This hypothesis is based on our observation that FAP expression in melanocytic skin tumors was also detected on the surface of melanocytes in 30% of melanocytic nevi assayed. A significantly larger number of nevi will need to be assayed for FAP expression, in order to link expression in nevi with clinical melanoma progression and prognosis. In contrast, FAP expression was not found on melanoma cells from primary and metastatic melanomas, consistent with previous observations obtained in *in vitro* melanoma models (Rettig *et al*, 1993). The role of FAP on melanocytic nevus

cells, as well as its downregulation on melanoma cells – which is in contrast to many other proteases, including matrix metalloproteinases and uPA (Hofmann *et al*, 2000; Ruiter *et al*, 2002) – is currently not understood. Further insight into the role of FAP on melanoma *versus* stromal cells may be provided by further dissection of growth and/or differentiation signals leading to the activation of FAP in different cell types, such as transforming growth factor β or retinoic acid, as previously suggested (Rettig *et al*, 1994). In melanoma cells, FAP appears to form heterodimers with the related protease DPP-IV/CD26, which is also downregulated in the *in vitro* models previously analyzed (Rettig *et al*, 1993). Wesley *et al* (1999) reported that reexpression of DPP-IV/CD26 in melanoma cells at levels expressed by normal melanocytes leads to a loss of tumorigenicity and change in phenotype that is characteristic of normal melanocytes. Further functional studies will be required to determine whether FAP and DPP-IV/CD26 have similar or even partially redundant functions in cell survival and transformation of melanocytes.

Interestingly, we observed strong protein expression of FAP in patterned structures in a subset of the melanoma metastases analyzed in this study. As the FAP-positive networks described in this report are negative for endothelial as well as melanoma cell surface markers, but appear to have close contact to tumor blood vessels, we propose that they are most probably derived from stromal fibroblasts. Several studies have previously identified patterned structures in the melanoma stroma. In uveal melanoma, using a modification of the periodic acid–Schiff reaction, Folberg *et al* (1992) identified so-called "vascular patterns", some of which were associated with an unfavorable prognosis. Based on more recent studies (Foss *et al*, 1997; Clarijs *et al*, 2002), the current opinion is that the "vascular patterns" in uveal melanoma represent fibrovascular stromal septa, but not microvessels expressing endothelial markers (Ruiter *et al*, 2002). Several groups (McDonald *et al*, 2000; Clarijs *et al*, 2002) were unable to detect the "vascular channels" described by Maniotis *et al* (1999) as erythrocyte-filled spaces lined by melanoma cells communicating directly with endothelium-lined microvessels. Instead, they suggested that the fibrovascular septa may act as an extracellular matrix meshwork that provides a medium for diffusion of soluble factors. Recently, Warso *et al* (2001) identified fibrovascular stromal patterns in primary cutaneous melanoma that resembled those observed in uvea melanoma. Architectural variations in and around cutaneous melanoma appear to include several patterns, such as nodular, micronodular or nested, mixed nodular/micronodular, and desmoplastic, several of which form typical "loop" and "network" structures (Banerjee and Harris, 2000; Ruiter *et al*, 2002).

Ultimately, to pinpoint the importance of FAP for melanoma onset and/or progression, we may have to await the results of mouse tumor models or, more likely, studies with highly selective inhibitors of FAP. Stromal therapy is increasingly viewed as an attractive approach to cancer prevention and intervention (Liotta and Kohn, 2001). Selective ablation of FAP-positive stromal fibroblasts will constitute a promising approach to demonstrate the contribution of stromal fibroblasts to melanocytic skin tumors. In summary, FAP expression appears to be a highly specific marker for reactive tumor stromal fibroblasts of human epithelial cancers and, as demonstrated in this report, of melanocytic skin tumors. It remains to be seen whether stromal expression of FAP in highly patterned structures found in melanoma metastases will correlate with a poor prognosis.

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