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Differential Expression of Vinculin between Weakly and Highly Metastatic B16-Melanoma Cell Lines

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We previously reported on the altered expression of a third actin in mouse-B16 melanoma associated with malignant progression. While further investigating the relationship of cytoskeletal proteins to malignancy, we found that the expression of vinculin was higher in weakly metastatic B16-F1 cells than in highly metastatic B16-F10 cells. By Northern blot analysis, the mRNA expression of vinculin in B16-F1 was also shown to be higher than in B16-F10. Immunofluorescence staining showed a clear dotted distribution of vinculin in B16-F1, but only a weak and diffuse distribution in B16-F10. The dotted distribution tended to be larger in B16-F1 and when cultured on Matrigel and fibronectin than on laminin and type IV collagen. An alteration in the expression of vinculin was also observed in other cell systems. Vinculin was detected in both normal 3Y1 and in relatively weakly malignant transformed 3Y1 cell lines, while vinculin was either scarcely detected or not detected at all in more malignant cell lines. These results suggest that the suppression of vinculin is closely related to malignant progression in both the B16-melanoma and 3Y1 cell systems.

Key words: Actin — Vinculin — Cytoskeleton — Melanoma

It has been reported by a number of laboratories that the malignant transformation of eukaryotic cells is associated with a change of cytoskeletal organization.[1-5] We detected an acidic actin coexpressed with β, γ actins in mouse B16-melanoma; it was expressed in weakly metastatic B16-melanoma cell lines while it was either scarcely detected or not detected at all in highly metastatic ones.[6] We named the acidic actin βm actin (first tentatively named as A^X actin). The decrease in the expression of βm actin was accompanied by an increase in invasiveness and metastasis in several B16-melanoma cell lines besides B16-F1 and B16-F10.[7]

The expression of the βm actin in mouse B16-melanoma cell lines appeared to be correlated with an increase in the cytoskeletal organization.[8,9] Since cytoskeletal organization should be regulated by a number of related proteins in addition to actin, we further analyzed the expression of other cytoskeletal proteins, and found that vinculin was differentially expressed between weakly and highly metastatic cell lines.

Vinculin is a membrane-associated cytoskeletal protein, localized at the adhesive junction (cell to cell), and at the adhesion plaque (cell to substrate).[10,11] This protein seems to function as an intermediate component between transmembrane protein and actin fibers,[12] although the physiological function and the mode of interaction with molecules at the adherence junction and adherence plaque, such as integrin, talin, α-actinin and actin filament, remain to be clarified. In this paper, we describe the expression and distribution of vinculin among several cell lines with different degrees of malignancy.

MATERIALS AND METHODS

Cell lines Mouse B16-F1, B16-F10, B16-F1F, B16-F4F, and B16-F10F were used in this study; their origin and properties have been described previously.[7,13] B16-F1F, F4F and F10F cell lines were established by repeated *in vitro* and *in vivo* selection for highly metastatic cells from parent B16 melanoma in a similar manner to that of Fidler.[13] A rat embryo fibroblast cell line, 3Y1-B clone 1-6 (3Y1),[14] and derivative cell lines transformed by adenovirus, Ad12-3Y1-W5 (Ad),[14] by SV-40 virus, SV-3Y1-66 (SV),[14] by Rous sarcoma virus, SR-3Y1-1, 2, 3, (SR),[15] by v-H-*ras*, HR-3Y1-1, 2, 3, (HR),[16] by N-methyl-N′-nitro-N-nitrosoguanidine, NG-3Y1-1, 2, 3, 4 (NG)[17] and by Rous sarcoma virus plus v-*fos* (pFBJ-2), fos-SR-3Y1-202 (fos-SR-202)[18] have been previously described. Mouse B16-melanoma cell lines, and rat 3Y1 and transformed cell lines, were grown in Eagle's minimum essential medium and Dulbecco's modified Eagle's medium, respectively, and supplemented with 10% fetal bovine serum, bicarbonate-buffered (3.7 g/liter), in a humid atmosphere of 10% CO_2 and 90% air.

Western blot analysis The cells were harvested and treated with 9 volumes of ice-cold acetone. The dried acetone powder was dissolved in a sample buffer and analyzed with polyacrylamide gel electrophoresis in sodium dodecyl sulfate and Western blot immunostain-

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ing,[6] using anti-vinculin antibody (monoclonal VIN-11-5, Sigma Chem. Co., St. Louis, MO).

Northern blot analysis Total RNA was prepared from the cells by the guanidium isothiocyanate method,[19] electrophoresed and blotted onto nitrocellulose filters. The hybridization was performed as previously described, using a cDNA probe of mouse vinculin,[20] which was kindly donated by Drs. A. Ben-Ze'ev and B. Geiger, and labeled by the random primer method with ^{32}P-dCTP.

Cell staining Mouse B16-F1 and B16-F10 were cultured on cover slips (1 cm × 1 cm) coated with 10 μg of Matrigel, fibronectin, laminin, or type-IV collagen for 3 days. Cells were fixed with 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room

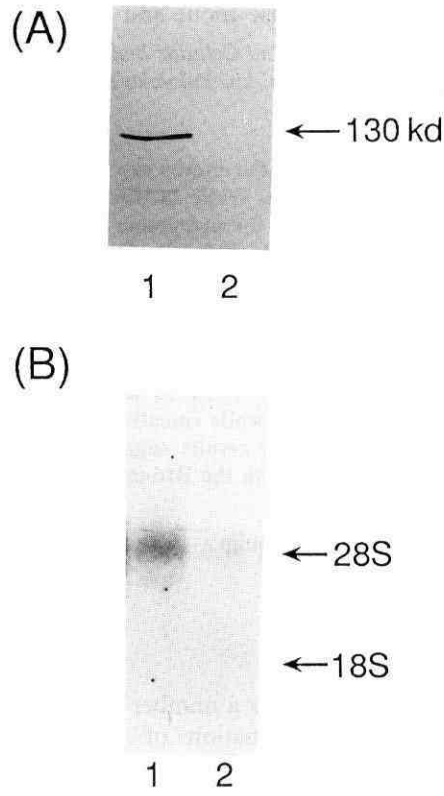

Fig. 1. (A) Western blot analysis for vinculin in B16-F1 and F10. Total cellular proteins (10 μg) of B16-F1 (lane 1) and F10 (lane 2) were separated by SDS-polyacrylamide gel electrophoresis and electroblotted. Western blots were stained with anti-vinculin antibody as described in "Materials and Methods." Arrows indicate the molecular weights of the bands calculated from the mobility of molecular weight markers; 130 kd indicates the molecular weight of vinculin. (B) Northern blot analysis for the expression of vinculin in B16-F1 and F10. Total RNAs (20 μg) of F1 (lane 1) and F10 (lane 2) were electrophoresed in 0.8% agarose gel and blotted. The hybridization with the ^{32}P-labeled vinculin DNA was carried out as described in "Materials and Methods."

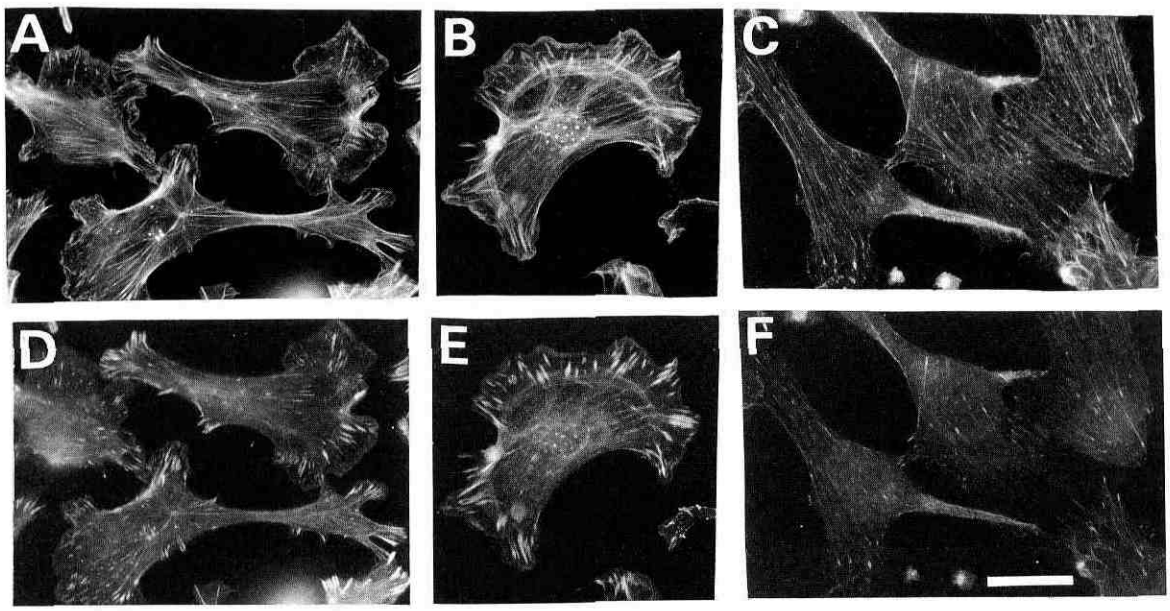

Fig. 2. Localization of actin stress fibers and vinculin. B16-F1 cells (A, B, D, and E) and F10 cells (C and F) were fixed with paraformaldehyde and permeabilized with Triton X-100. Double staining for actin stress fibers with FITC-conjugated phalloidine (A, B, and C) and for vinculin with rhodamine-conjugated second antibody (D, E, and F) were carried out as described in "Materials and Methods." Bar: 10 μm.

temperature and then were permeabilized with 0.1% Triton X-100 in PBS for 2 min. Double staining was first carried out with fluorescein isothiocyanate (FITC)-conjugated phalloidine (Wako Pure chemical Industries, Ltd., Osaka) for 30 min and washed with PBS. Secondly, anti-vinculin (mouse monoclonal VIN-11-5, Sigma Chem. Co.) diluted 1:1000 with PBS containing 0.1% blocking reagent (Boehringer Mannheim Yamanouchi Company) was poured over permeabilized cells or FITC-phalloidine-exposed cells for an additional 1 h at room temperature. After three washes of the cells with PBS, the cells were immersed in rhodamine-conjugated-goat anti-mouse Ig2a (ICN Immuno Biologicals, Lusie, IL) diluted 1:1000 with PBS containing 0.1% blocking reagent for 1 h at room temperature. The coverslips were rinsed with PBS and then mounted with a 9:1 mixture of glycerol and PBS containing 1% 2-mercaptoethanol. The fluorescence of the cells was then examined with a Zeiss Axio-scope and photographed on Kodak Tri-X-400 film.

RESULTS

A Western blot analysis indicated that vinculin was expressed highly in B16-F1, but not B16-F10 (Fig. 1A). To check the mRNA level of vinculin, we carried out a Northern blot analysis using mouse vinculin cDNA probe. A signal at the position corresponding to 28S was detected in B16-F1, while only a faint signal was observed in B16-F10 (Fig. 1B).

Fig. 2 shows the fluorescence staining of vinculin and stress fibers in B16-F1 and B16-F10. As previously described, B16-F1 had larger and thicker fibers than B16-F10.[7] The vinculin staining pattern showed large dotted patterns on the peripheral and internal positions of cells at the end of stress fibers as described for fibroblast cell lines.[10] The dotted area of B16-F1 was much larger than that of B16-F10, which was consistent with the results of the Western blot analysis. In B16-F10, the small dots of vinculin were detected at the terminals of the stress fibers. The extent of the dotted area of vinculin appeared to be correlated with the thickness of stress fibers present in the B16-melanoma cell lines.

Fig. 3 shows that the expression of vinculin in B16-F1 is more than that in B16-F10 cultured on various extracellular matrices, Matrigel, fibronectin, type-IV collagen, and laminin. The area of immunofluorescence of vinculin in B16-F1 tended to increase on Matrigel and fibronectin, although this phenomenon was unclear in the case of B16-F10.

To assess the relationship between malignancy of transformed cells and vinculin expression, we performed a Western blot analysis on the B16-F1F, B16-F4F, and B16-F10F, and 3Y1-transformed cell lines (Fig. 4). The metastatic ability, invasiveness and cytoskeletal disorganization of actin stress fibers of B16-F1F, B16-F4F and B16-F10F were augmented with an increase in the F number, while the expression of βm actin decreased with increase in the F number. In B16-F1F and B16-F4F, vinculin was expressed more than in B16-F10F. As for the 3Y1 cell system, normal 3Y1 cells, and NG-3Y1 cell lines, expressed large amounts of vinculin (Fig. 4). On the other hand, SR-3Y1, fos-SR-202 and Ad-3Y1 cells

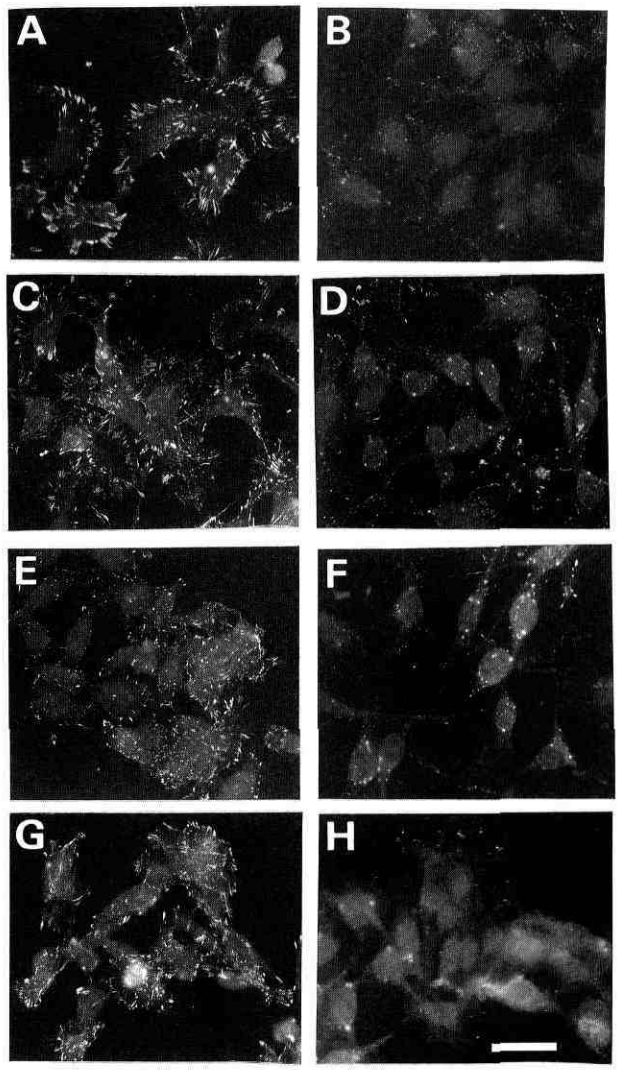

Fig. 3. Immunofluorescence microscopy of B16-melanoma cultured on various extracellular matrices. B16-F1 (A, C, E, and G) and F10 (B, D, F, and H) were cultured on Matrigel (A and B), fibronectin (C and D), laminin (E and F), and type-IV collagen (G and H) coated on cover slips for 3 days. All cells were stained with anti-vinculin and rhodamine-conjugated second antibody as described in "Materials and Methods." Bar: 10 μm.

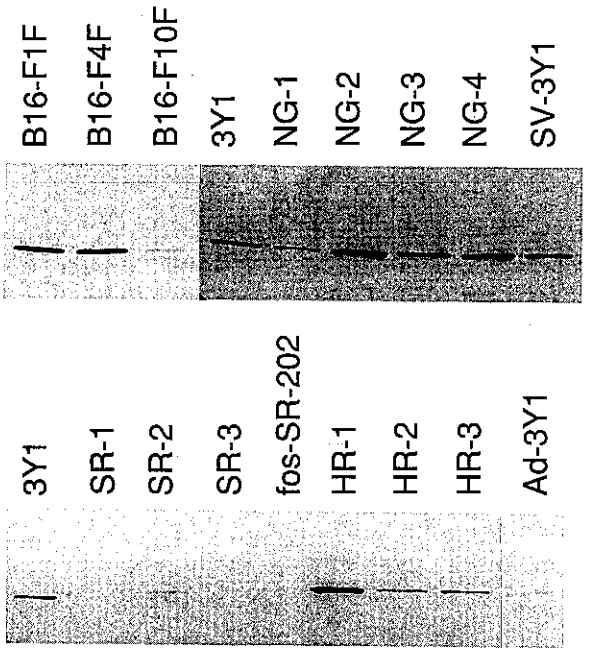

Fig. 4. Expression of vinculin in transformed cell lines. Total cell proteins (10 μg) were used for Western blot analysis as described in "Materials and Methods." B16-F1F, F4F, and F10F are B16-melanoma cell lines and possess different metastatic ability. 3Y1 is a normal rat 3Y1 fibroblast cell line; NG-1, -2, -3, and -4 are 3Y1 cell lines transformed by N-methyl-N'-nitro-N-nitrosoguanidine; SV-3Y1 by simian virus 40; SR-1, -2, and -3 by Rous avian sarcoma virus; fos-SR-202 is an SR-2 cell line bearing a transferred v-*fos* oncogene; HR-1, -2, and -3 have v-H-*ras* oncogene; Ad-3Y1 carries adenovirus type 12.

expressed either no or only scant amounts of vinculin. Consistent with the results of Western blot analysis, vinculin was more frequently detected as largely dotted patterns in 3Y1, NG-, SV-3Y1 cells than in SR-3Y1 cells by immunofluorescence staining using anti-vinculin antibody (data not shown).

DISCUSSION

B16-F1 and weakly metastatic B16-melanoma cell lines have relatively well organized stress fibers, as compared with highly metastatic cell lines, such as B16-F10. Stress fibers consist of a number of cytoskeletal proteins, such as actin, tropomyosin, myosin and α-actinin, which are involved in the regulation of cell morphology and motility. An alteration in either the amount or ratio of the cytoskeletal proteins seems to be responsible for the malignant phenotypes of neoplastic cells. We first noted the presence of an acidic actin (βm actin)[6,7] as an antimetastasizing gene in B16-melanoma cells, and the actin appeared to increase the amount of actin stress fibers, leading to a decrease in cell motility and invasiveness. The structural analysis of βm cDNA indicated that βm has one amino acid exchange at the 28th amino acid as compared with the hitherto established mouse β actin (βm, leucine; β, arginine) as well as having several differences of nucleotide sequence in the untranslated 5′ and 3′ regions.[8] When the cDNA of βm was transferred into B16-F10, the number of stress fibers increased in the transfectants.[9]

Table I. Differential Expression of Vinculin Correlated with the Expression of Third Actins among Various Cell Lines

Cell line	Organization of			Malignancy
	third actin	stress fibers	vinculin	
B16-F1	++βm	good	+	weakly metastatic
F10	−	poor	−	highly metastatic
F1F	++	good	+	weakly metastatic
F4F	+	good	+	moderately metastatic
F10F	+−	poor	−	highly metastatic
3Y1	+α-SMA	good	+	normal
SV-3Y1	+	good	+	anchorage-dependent
Ad-3Y1	−	poor	−	anchorage-independent
SR-3Y1	−	poor	−	metastatic
HR-3Y1	+−	intermediate	+−	tumorigenic, weakly metastatic
fos-SR-3Y1	−	poor	−	highly metastatic
NG-3Y1	+	good	+	weakly tumorigenic

The characteristics of the cell lines were described in previous reports.[6,7,13-18,22] The expressions of the third actins and vinculin were assessed by means of Western blot analyses and the stress fibers were examined with rhodamine-phalloidine staining as described in "Materials and Methods." α-SMA; smooth muscle α actin.

A further search for the molecular difference of stress fibers between B16-F1 and B16-F10 yielded evidence of an inverse correlation of vinculin expression with disorganization of the actin stress fibers, invasiveness and the metastatic potential, as seen for βm actin. Recently, Ben-Ze'ev *et al.* reported the induction of vinculin in Balb/c 3T3 fibroblasts stimulated with serum and several growth factors, and the formation of a dotted distribution in the adhesion plaque on fibronectin.[20] In the case of B16-melanoma, weakly malignant B16-F1 formed a large dotted pattern on the extracellular matrix, but highly malignant B16-F10 showed a very small dotted distribution. Although B16-F1 cells adhere to plastic and extracellular matrix, the amount and dotted pattern of vinculin is altered, which may cause functional differences in both the adhesive phenotypes and malignancy. Vinculin is phosphorylated by tyrosine kinase, $pp60^{src}$.[21] Because the anti-vinculin antibody reacted with both the phosphorylated form and the non-phosphorylated form of vinculin, the present Western blot analysis indicated a quantitative difference in the amount of vinculin protein. An investigation is under way to determine whether there are any qualitative differences in vinculin among cell lines of different malignancies.

In our previous work,[22] we noted that α actin was expressed in 3Y1 and the expression was changed in association with malignant transformation and/or progression: α actin has been detected in 3Y1, HR-, SV- and NG-3Y1 cell lines, but not in SR-, fos-SR-202, or Ad-3Y1 cell line. The former cell lines are low in anchorage independency, tumorigenicity or metastasis as compared with the latter ones, as summarized in Table I. In particular, HR-3Y1 cell line, an HR-1 clone expressing α actin and vinculin, is less tumorigenic than other clones (data not shown). In rat 3Y1 cell lines NG, HR and SV expressing α actin and vinculin, stress fibers were more frequently observed than in other transformed cells. The apparent correlation of well organized stress fibers with the expression of a third actin (βm) and vinculin has already been mentioned regarding mouse B16-melanoma. Thus, α actin and βm actin are likely to have a function of increasing stress fibers in cooperation with vinculin.

The investigation of the dynamic regulation in actin stress fibers by actin and actin-associating proteins, such as vinculin, would be useful for understanding not only the regulation of the cytoskeletal system but also the molecular mechanisms of malignant progression.

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