Newly identified type of β actin reduces invasiveness of mouse B16-melanoma

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Low metastatic parent B16 melanoma and isolated B16-F1 cell lines have a third actin designated as βm(A^X:previously). βm actin is scantily or not at all detected in highly metastatic cell lines, such as B16-F10 and BL6. To directly assess the physiological role of βm in phenotypic changes of B16 melanoma, we transfected expression plasmids of βm into B16-F10 cells. The actin expressed in the transfectants is located largely in cytoskeletal fractios. The transfectants exhibited a larger number of stress fibers and a lower invasiveness than did the recipient cells. Thus, βm actin plays an important role in the organization of actin stress fibers, the result being a decrease in invasiveness of B16 melanoma.

Actin; DNA transfer; Stress fiber; Cytoskeleton

1. INTRODUCTION

A third actin (βm) detected in mouse B16 melanoma is a newly identified type of cytoskeletal actin [1,2]. The amino acid sequence of this actin, determined by the nucleotide sequence of the cDNA, differed from β actin at the 28th amino acid (β, arginine; βm, leucine) (Fig. 1A), and at five amino acids from γ actin. In the nucleotide sequence of βm cDNA, there were two sites with one base change, one site of one base insertion and four regions of deletion compared with hithertofore known mouse β actin [3]. Therefore, βm actin is closely related to β actin, which consists of a multiple gene family containing active and pseudogenes [4,5]. βm was co-expressed with β and γ actins in parent B16 melanoma and the B16-F1 cell lines, both of which are low metastatic, yet little or not at all in the highly metastatic B16-F10 cell line, indicating inverse correlation of βm actin expression and metastatic ability of B16-melanoma [1,6]. Previously, mutant β actin which has one amino acid exchange at the 244th amino acid (mutant β, aspartic acid; β, glycine) was reported in reference of tumor progression and disorganization of cytoskeleton [7–9]. Leavitt et al. [10,11] showed that the expression of mutant β actin in human fibroblast KD cells caused tumor progression, by gene transfer experiments of mutant β actin DNA. The site of amino acid exchange is different between βm and mutant β actin, which may correspond to inverse function in the organization of cytoskeleton and tumor phenotypes. To investigate the physiological role of βm actin, we transfected βm actin cDNA into highly metastatic B16-F10 cells expressing no βm actin and analyzed the nature of the transfectants.

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2. MATERIALS AND METHODS

2.1. *Cells and DNA transfection*

B16-F1 and F10, the origin and properties of which were described by I.J. Fidler [12], were grown in Eagle's minimum essential medium (MEM, Nissui) supplemented with 10% fetal bovine serum (FBS, Gibco), bicarbonate-buffered (3.7 g/liter) in a moist atmosphere of 10% CO_2 and 90% air. An expression vector was constructed from βm actin cDNA and 5′ UTR region containing human β actin promoter [13] and pUC18 (pHβA). Another one was constructed from pBR322 plasmid containing mouse mammary tumor virus LTR [14], inducible by dexamethasone, βm actin cDNA and Eco gpt gene (pMMA). As the Eco gpt gene was not effective as a genetic marker in B16-F10 cells, pSV2-neo (1 μg) was co-transfected with the plasmid (10 μg) into B16-melanoma F10 cells (10^5 cells/dish) by the calcium phosphate precipitation method. G-418 (600 μg/ml) selection was performed for 7 days and independent clonal $G418^r$ cells were isolated. Total proteins and Triton X-100 insoluble cytoskeletal fractions were obtained from 10^7 transfectants, and then two dimensional gel electrophoresis and Western blot analysis, and rhodamine-phalloidin staining was performed as described previously [6].

2.2. *In vitro invasion analysis*

In vitro invasion assay was carried out by the method of Albini et al. [15] using polycarbonate filters, with 8 μm pore size (Nuclepore, CA), which were coated with 50 μg of the basement membrane matrigel.

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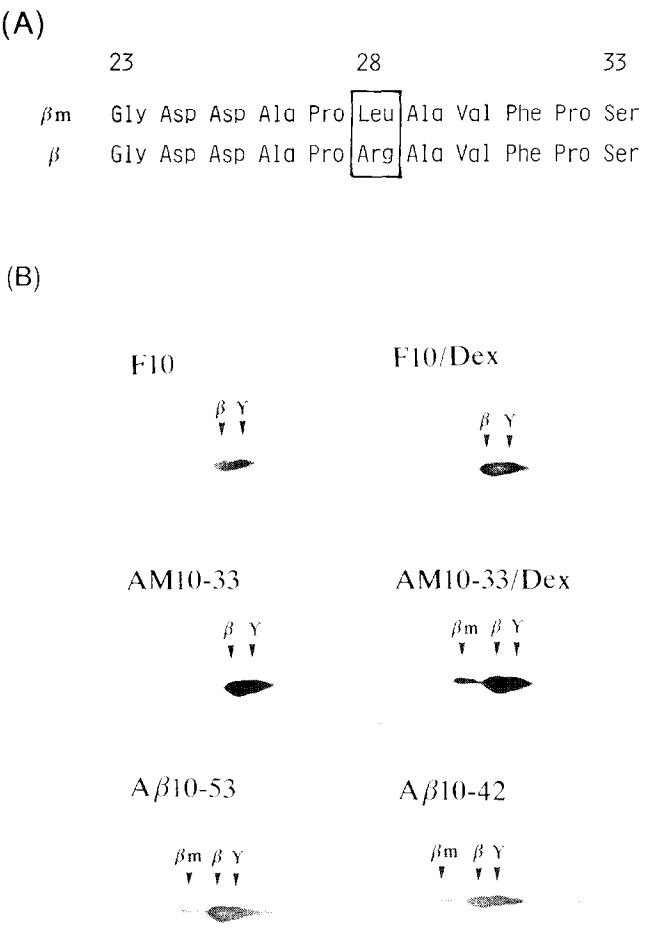

Fig. 1. (A) The amino terminal side sequences of mouse βm actin containing a different amino acid from β actin, whereas the carboxyl side sequences are the same as β actin. (B) Expression of βm actin in the transfectants (B16-F10) carrying βm cDNA grown with or without 5 μM dexamethasone for 3 days. Cells (10^7) were harvested and treated with 9 vols of cold acetone. The acetone powder was dissolved in O'Farrell's lysis buffer, and the sample corresponding to 10^6 cells was analyzed with two dimensional gel electrophoresis and Western blot immunostaining, using anti-actin antibody and peroxidase conjugated second antibody. The AM10-33 clone is a clonal transfectant with pMMA, the Aβ10-53 and 42 were independent clones transfected with pHβA.

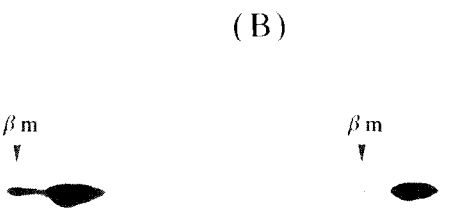

Fig. 2. Distribution of βm actin in the transfectants. 10^7 cells were harvested and extracted with PBS-buffer containing 0.5% Triton X-100 at 4°C for 10 min, and Triton X-100 insoluble cytoskeletal fractions (A) and soluble fractions (B) were prepared and dissolved in equal volumes of lysis buffer. The samples corresponding to 10^6 cells were used for two dimensional gel electrophoresis and Western blot analysis with anti-actin antibody.

3. RESULTS

The transfectants were cloned and the actin expression was examined by Western blot analysis, using anti-actin antibody. Independent clones, Aβ10-42 and Aβ10-53 carrying pHβA plasmid expressed βm actin (Fig. 1B). In clone AM10-33 carrying pMMA, βm actin was induced only when cultured with 5 μM dexamethasone, but not without dexamethasone (Fig. 1B). Exogenous plasmid DNA containing βm cDNA in these three clones was detected as extra DNA bands by Southern blot analysis (data not shown). Since B16-F10 cells did not express βm actin irrespective of treatment with dexamethasone (Fig. 1B), the βm actin expression in B16-F10 transfectants was derived from the exogenous DNA plasmid. Densitometric measurement showed that the relative proportion of β to γ actin (β/γ) was unchanged between F10 and transfectants (data not shown).

To investigate the distribution of βm actin in the transfectants, we isolated the cytoskeletal fractions and Triton X-100 soluble fractions. Fig. 2A shows that βm actin expressed in AM10-33 cultured with 5 μM dexamethasone located more in cytoskeletal fractions than in the Triton X-100 soluble fractions (Fig. 2B) as compared with β and γ actin. The amounts of β and γ actins in cytoskeletal fractions were slightly larger than in soluble fractions, but the ratio of β/γ actin in both fractions was much the same. In other transfectants, Aβ10-42 and Aβ10-53, the same phenomena were found (data not shown).

We investigated the cytoskeletal organization of B16-F10 transfectants expressing βm actin. AM10-33 cultured with dexamethasone and Aβ10-53 showed more actin stress fibers than the non-induced cells and recipient B16-F10 cells (Fig. 3). The induction of βm and increase in actin stress fibers were reversible phenomena depending on dexamethasone treatment (data not shown).

To investigate the effect of βm actin on cell invasiveness, we carried out an in vitro analysis using matrigel-coated filters. AM10-33 cells cultured under βm inducible conditions showed a lower penetration than did the B16-F10 cells, irrespective of the treatment of the recipient with dexamethasone (Fig. 4A). Aβ10-53 and Aβ10-42 cells, constitutively expressing βm, also showed lower invasiveness than did B16-F10 cells. The invasiveness of Aβ10-53 and Aβ10-42 decreased to much the same level seen in the B16-F1 cells (Fig. 4B). On the other hand, F10 transfected pSV2-neo (Neo10) showed no significant reduction in in vitro invasiveness.

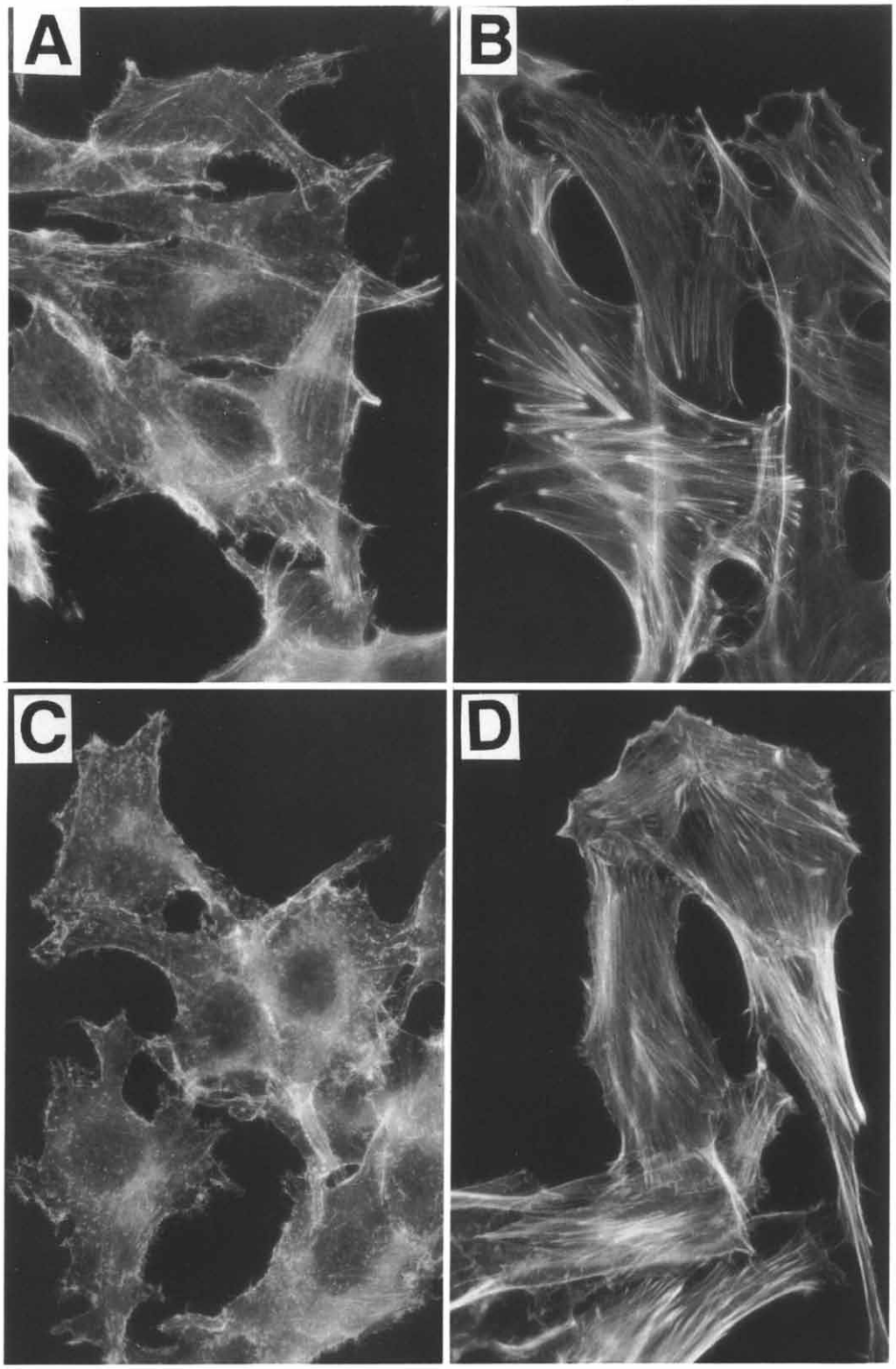

Fig. 3. Organization of actin stress fibers associated with expression of βm actin. The cells AM10-33 were grown without (A) or with (B) 5 μM dexamethasone for 3 days. C, D indicated the staining of F10 and Aβ10-53, respectively. The cells were cultured on cover glasses and fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature, then were made permeable with 0.1% Triton X-100 in PBS for 2 min. Staining with rhodamine-conjugated phalloidin was carried out for 30 min. After a wash with PBS, the preparations were mounted with 90% glycerol–10% PBS containing 0.1% 2β-mercaptoethanol and observed under a fluorescence microscope (Olympus, Tokyo).

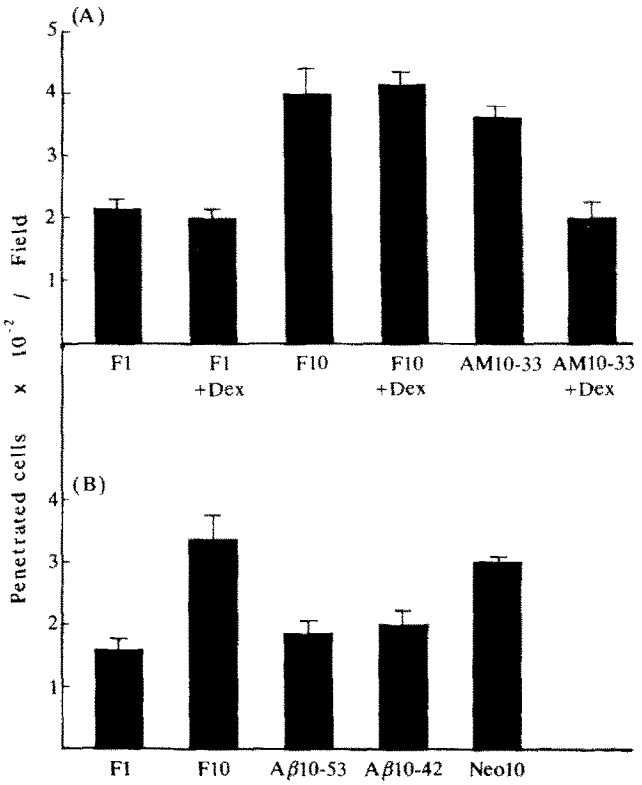

Fig. 4. In vitro invasion assay of βm actin transfectants. B16-F1(F1), B16-F10(F10), and AM10-33 were cultured with (+Dex) or without 5 μM dexamethasone for 3 days in (A). In (B), F1, F10, Aβ10-53, Aβ10-42 and pSV2-neo-transferred F10 (Neo10) were cultured and harvested. Cells (3×10^5), suspended in DMEM containing 0.1% BSA, were added to the upper compartment of Boyden chambers and incubated at 37°C in 10% CO_2 for 4 h. The lower compartments of the chambers were filled with conditioned medium, obtained by incubating NIH 3T3 cells for 24 h in serum-free medium containing 250 mg ascorbate per liter. The number of the cells penetrated through the matrigel coated filters was counted. Each column indicates the mean of triplicate samples; bar, SE. The number of cells per field was evaluated by Student's t-test. P value: AM10-33 + Dex versus AM10-33 and F10 + Dex, <0.01; AM10-33 versus F10 + Dex, >0.05; Aβ10-53 and Aβ10-42 versus F10, <0.01; Neo10 versus F10, >0.05.

4. DISCUSSION

We reported that the B16-F1 cell line, expressing βm actin at a high level, has a larger number of actin stress fibers and a lower metastatic potential than did the B16-F10 cell line not expressing βm actin [6]. βm actin was incorporated into the cytoskeleton of B16-F1 cells as seen with β and γ actin [6], and had the DNase I binding potential seen in other native actin species [2]. Induction of the βm expression derived from exogenous βm cDNA was associated with increase in number of actin stress fibers and decrease in the in vitro invasion across matrigel-coated filters. Thus, βm actin seems to promote construction of the stress fibers, leading to reduction of the cell motility and/or flexibility. Since there was no difference in the amount of $\beta+\gamma$ actins between F1 and F10, βm actin is an additive population in B16-F1. High efficiency of incorporation of βm actin into cytoskeletal fractions in the transfectants appears to indicate higher competence of βm actin to form actin filaments than β and γ actins. We previously observed much the same efficiency in incorporation of βm, β and γ actins into cytoskeletal fraction of B16-F1. The high ratio of βm actin in the cytoskeletal fraction of the transfectants might be due to intracellular conditions, which are advantageous to nuclear formations, incorporation into actin filaments and/or interaction with actin-associating proteins. A mutant β actin, which has one amino acid exchange (glycine, β; aspartic acid, mutant β at amino acid 244), had less polymerizing activity in vitro and in vivo, leading to disorganization of the actin stress fibers in KD-fibroblast [7–9]. Leavitt et al. noted that transfer of the mutant β actin into a transformed but low tumorigenic cell line increased the malignancy and altered the organization of the microfilaments [10,11]. These inverse biochemical and biological functions of this mutant β actin against βm actin in B16 melanoma may be attributed to the difference in the site of amino acid change. Regardless of low expression of βm actin in B16-F10 transfectants, the integrity of actin stress fibers increased and the malignancy decreased, showing efficient biological functions of βm actin on such cellular phenotypes. It remains to be examined whether the integrity of actin stress fibers is restored by other exogenously transferred actin isomers, such as β, γ, or α actin.

Changes of cytoskeletal proteins are associated with malignant transformation and/or progression of various cells and it is widely assumed that these changes are involved in the alteration of cell morphology, flexibility and motility [16–19]. In addition to our finding for βm actin in B16 melanoma, there are reports on the depression of a third functionally normal actin associated with cell transformation. In chick embryo cells [20], and rat and mouse fibroblast cell lines [21], α type actin was either decreased or disappeared in the transformants. We also found that α actin identified as the smooth muscle type was expressed in a normal rat 3Y1 cell line but decreased or was absent in the malignant counterparts [22]. The α actin of smooth muscle type detected in human pigment tissues tends to decrease in malignant melanoma tissues [23]. Thus, alteration in expression of cytoskeletal actin often accompanies malignant transformation and/or progression. However, the role of the α type actin in non-muscle cells remains to be investigated. The functional differences among βm, β, γ and α actin are an interesting subject. Because there is no method to reproducibly separate actin isomers maintaining the native functions, a new method for isolation has to be developed.

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