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Altered Expression of a Third Actin Accompanying Malignant Progression in Mouse B16 Melanoma Cells

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The expression of actin was examined and compared in several mouse B16 melanoma cell lines with different metastatic ability, by the use of two-dimensional gel electrophoresis or horizontal isoelectric focusing. In the mouse B16 melanoma cell lines, the expression of newly found A^X actin (Mr = 43,000, pI = 5.2) decreased with the increase in *in vitro* and *in vivo* selection cycles (F number) for high-metastatic cells. On the contrary, the metastatic ability of each mouse cell line, assessed by lung colony-forming ability following iv administration, increased with increase in the F number. The half life of A^X actin was much the same as that of β- and γ-actin and the different expressions of A^X actin between the low- (F = 1) and high-metastatic (F = 10) cell lines were attributed to differences in the rate of synthesis but not in the decay rate of A^X actin. The A^X actin was incorporated into the cytoskeletal fraction with the same efficiency as β- and γ-actin. The invasiveness of the cells, assessed *in vitro* using matrigel, was increased with the decrease in A^X expression. The actin stress fibers, observed staining with rhodamine-conjugated phalloidin, were organized better in a low-metastatic cell line (F = 1) than in a high-metastatic one (F = 10). These results suggest to us that depression of A^X actin is involved in disorganization of the cytoskeletal system, the cellular flexibility and motility are enhanced and there is a consequent increase in the invasiveness and metastatic potential.

Key words: Actin — Metastasis — B16 melanoma

It is widely assumed that quantitative and/or qualitative changes in gene and protein expression accompany cell malignant progression. With regard to phenotypes in neoplastic cells, we have directed our attention to changes in the expression of cytoskeletal proteins, with special reference to actin, a protein which is the main component of microfilaments controlling cell morphology and motility.[1,2] As one of the most common biological characteristics in neoplastic cells is alteration of cell morphology and motility,[3,4] quantitative and/or qualitative changes in actin proteins in the cells of a defined malignancy are of interest.

Actin is an abundant and highly conserved protein,[5] and six isoforms have been identified.[6] In non muscle cells of vertebrates, β- and γ-actin are usually expressed. Recently, we found a different type of actin (A^X actin) coexpressed with β- and γ-actin in B16-melanoma cells, which disappeared in highly metastatic cell lines, such as B16-F10 and B16-BL6.[7]

To assess the correlation of A^X actin expression with the metastatic potential and invasiveness of B16 melanoma, we examined the expression of A^X actin in several B16 melanoma cell lines with an intermediate metastatic ability, in addition to extremely low- and high-metastatic cell lines. We found that the expression of A^X actin was inversely correlated with increase in lung-colony forming ability and invasiveness *in vitro*, and with decrease in organization of the actin stress fibers.

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MATERIALS AND METHODS

Cell culture Parent B16 melanoma, B16-F1, B16-F10 and B16-BL6, the origin and properties of which were described by I. J. Fidler,[8] were kindly provided by Dr. T. Tsuruo, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo. Other B16-melanoma cell lines, independently isolated through *in vitro* and *in vivo* selection for high-metastatic cells, were donated by Fujisawa Pharmaceutical Co. Ltd., Osaka. The *in vitro* and *in vivo* selection involved formation of lung metastasis by intravenous injection of *in vitro*-cultured melanoma cells, transfer of the formed lung colony into *in vitro* culture, and the repetition of this process. These lines were tentatively designated as B16-F1F, F2F, F3F ··· F10F, for the present experiments; each number corresponds to the number of selection cycles. All cells 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.

Preparation of cellular proteins for electrophoresis Preparation and fractionation of cellular proteins were done as described.[7] The subconfluent cell monolayers in ϕ 60 mm Petri dishes were rinsed with cold Dulbecco's

phosphate-buffered saline. Solubilization of cellular proteins was carried out for 5 min at 4°C with 0.5 ml of extraction buffer: 1% Triton X-100 (v/v), 10 m*M* Tris-HCl, pH 7.8, 10 m*M* $MgCl_2$, 30 m*M* KCl, 0.01 *M* 2-mercaptoethanol. The cytoskeletal proteins were prepared from the residue of Triton-soluble proteins. The insoluble residue was collected from the surface of dishes by scraping with a rubber policeman.

The Triton-insoluble residues were washed with a low-Triton (0.1%) extraction buffer, other components of which were the same as in the 1% Triton extraction buffer. The preparations were then pelletted by centrifugation at 10,000*g* for 5 min at 4°C. The lysis buffer of O'Farrell[9] was added to the pellet and the preparation was then dissolved by vigorous shaking at room temperature for 10 min. After centrifugation (10,000*g* for 2 min) of the lysed protein solution, the supernatant was collected as the cytoskeletal fraction and stored at −80°C.

Total proteins were also prepared as follows. The washed monolayers of the cells were collected and extracted three times with acetone chilled at −20°C. The insoluble pellet was dried and dissolved with O'Farrell's lysis buffer[9] by shaking vigorously at room temperature for 10 min, followed by centrifugation at 10,000*g* for 2 min. The supernatant was stored in the form of total protein fractions at −80°C.

Two-dimensional gel electrophoresis and horizontal isoelectric focusing For analysis of cellular proteins, two-dimensional polyacrylamide gel electrophoresis and isoelectric focusing in thin layers of polyacrylamide gels were used. In both vertical and horizontal isoelectric focusing, polyacrylamide gels consisting of the same components were prepared.[9]

The polyacrylamide concentration of the separating gel in the second dimension was 11%. Whenever vertical isoelectric focusing gels were subjected to a second sodium dodecyl sulfate-polyacrylamide gel electrophoresis, a small well was made at one side of the stacking gels for molecular markers or authentic actin samples. After electrophoresis, gels were stained with 0.15% Coomassie Blue, 50% methyl alcohol and 10% acetic acid for a few hours and then destained with 7.5% methanol and 7.5% acetic acid for about 15 h.

Horizontal isoelectric focusing was done using a Pharmacia flat bed apparatus (FBE 3000) and a Pharmacia electrophoresis constant power supply (ECPS 3000/150). Running conditions were as follows: constant power of 15 W, 100 Vh for the prerun, and a constant power of 30 W, 3000 Vh for focusing proteins at 15°C. After the isoelectric focusing, the gels were fixed with 4% sulfosalicyclic acid and 12.5% trichloroacetic acid for 1 h and stained with 0.04% Coomassie Blue R-250, 27% isopropanol, 10% acetic acid, and 0.5% $CuSO_4$ for 6 to 15 h. The gels were destained by immersion in 12% isopropanol, 7% acetic acid and 0.5% $CuSO_4$ for 6 to 15 h and then 7% actic acid and 5% methanol for 24 h. Densitometric measurements were made using a BIO-RAD video densitometer (model 620).

Western blotting and immunostaining Following the two-dimensional polyacrylamide gel electrophoresis, portions of the gels containing actins were cut out and equilibrated with 25 m*M* Tris, 192 m*M* glycine, and 20% methanol for 0.5 h at 4°C. The proteins in the polyacrylamide gels were transferred to nitrocellulose with the above buffer at room temperature and 0.8 mA/cm^2 for 2 h, according to Kyhse-Andersen.[10]

Immunostaining was done according to Hawkes *et al.*,[11] with a slight modification. Protein-blotted nitrocellulose sheets were immediately immersed in 50 m*M* Tris HCl, pH 8.0/0.9% NaCl (TBS), containing 0.1% Tween 20 and 1% BSA and then shaken gently at room temperature. The pretreated nitrocellulose sheets were then incubated for 3 h at room temperature in a plastic bag containing a small volume (50 $\mu l/cm^2$ of filter) of 500 to 1,000 times diluted mouse monoclonal anti-actin antibody (Amersham) in 0.1% Tween 20 and 0.1% BSA/TBS (reaction buffer). After washing the sheets with cold reaction buffer, we immersed them in 20 to 100 ml of 10 μl/ml peroxidase-conjugated goat anti-mouse immunoglobulins (Ig A, G & M, Cappel-Worthington) in the reaction buffer for 1 h. After removal of the second antibody in the same manner as described for washout of the first antibody, development was carried out at room temperature with 0.38 mg/ml 4-chloro-1-naphthol and 0.01% H_2O_2 in 50 m*M* Tris-HCl, pH 7.5. The developed filters were washed with running tap water and dried in room air.

Assay for experimental metastasis Gently trypsinized cells of several B16 melanoma cell lines were suspended in Hanks' balanced salt solution containing 1% mouse (C57BL/6) serum.[12] Metastatic ability of the cell lines was evaluated by iv administration of 1×10^5 viable cells/0.5 ml into the tail vein of female C57 BL/6 mice. The mice were killed 21 days later and the lungs were examined for colonies visible on the surface.

***In vitro* invasion assay using matrigel** The assay of *in vitro* invasiveness was done as described by Albini *et al.*[13] The basement membrane matrigel, an extract of the Englebrecht-Holm-Swarm (EHS) tumor, was prepared according to Kleinman *et al.*[14] Polycarbonate filters, 8 μm pore size (Nuclepore, California), were coated with 50 μg of the prepared basement membrane matrigel per filter, and placed in Boyden chambers. Cells (3×10^5/ml), suspended in DMEM containing 0.1% BSA, were added to the upper chamber. The lower compartments of the Boyden 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 cells were incubated at 37°C in 10% CO_2 for 5 h and those remaining on the upper surface of the filters were mechanically removed. The cells on the reverse side of the filters were fixed in methanol and stained with hematoxylin/eosin. The number of the cells which penetrated the filters was counted in 10 fields under a 200-fold magnification and each assay was done in triplicate.

Determination of synthesis and decay of A^X actin Cells in MEM containing 10% FBS were plated at 2×10^5 per ϕ 60 mm Petri dish and grown for 48 h at 37°C in 10% CO_2. Culture medium was replaced with methionine-free MEM to which 90 μCi of [^{35}S]methionine was added.[7] To assess the synthesis of actin, the cells were harvested at 30 min, 60 min and 120 min after the start of incubation and an acetone powder form of the cells was prepared, as described. The cell powder was dissolved in 100 μl of the lysis buffer of O'Farrell[9] and the total proteins were analyzed using two-dimensional gel electrophoresis. Actin spots of the Coomassie Blue stained gel were cut out, expanded with 1 ml of NCS tissue-solubilizer (Amersham) overnight at 50°C and neutralized with acetic acid before measuring radioactivity in the scintillant. To determine the half life of actin, the cells labeled with [^{35}S]methionine for 2 h were further incubated with fresh medium but without radio-labeled methionine. After 10 h and 20 h of further incubation, the cells were harvested and the radioactivity of each actin spot on two-dimensional gels was measured, in the same manner as described above.

Staining actin stress fibers Actin filaments of the cultured cells were stained at room temperature. Cells grown on glass coverslips were fixed with 3.7% formaldehyde in PBS for 30 min at room temperature, and then made permeable with 0.1% Triton X-100 in PBS for 2 min. Staining with rhodamine-conjugated phalloidin (Wako Pure Chemical Industries, Ltd., Osaka) was carried out for 30 min. After being washed with PBS, the cells were mounted with 90% glycerol containing 0.1% 2-β-mercaptoethanol and then observed under a fluorescence microscope (Olympus, Tokyo).

RESULTS

Comparison of expression of A^X actin between mouse B16-F1F and B16-F10F Figure 1 shows Coomassie Blue-stained two-dimensional gels of total and cytoskeletal proteins from B16-F1F and B16-F10F. In the patterns of total and cytoskeletal proteins (Fig. 1),

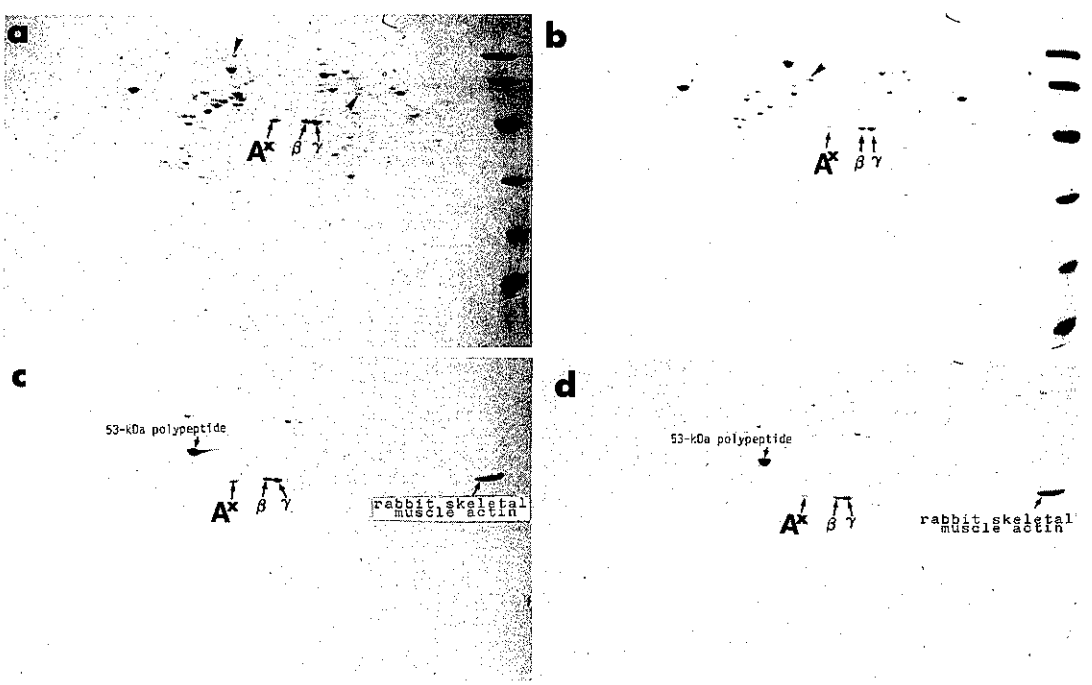

Fig. 1. Coomassie Blue-stained two-dimensional gels of total and cytoskeletal proteins from B16 melanoma cell lines. The acidic band is towards the left. Total polypeptides: a, B16-F1F; b, B16-F10F. Six bands on the right side are molecular size markers (from the top, 94 kDa, 67 kDa, 43 kDa, 30 kDa, 20.1 kDa, 14.4 kDa). Cytoskeletal polypeptides: c, B16-F1F; d, B16-F10F. These cell lines were isolated by Fujisawa Pharmaceutical Co. Ltd.

actin, confirmed by Western blot analysis using anti-actin monoclonal antibody (Fig. 2), was identified as one of the main spots, at 43,000 daltons and at pI 5.2–5.3. While the amount of A^X actin expressed in B16-F1F (Fig. 1a) was much the same as that of β- or γ-actin (A^X:β:γ= 1:1:1), the relative amount of A^X actin with respect to β- or γ-actin in B16-F10F (Fig. 1b) was decreased. The main spots identified in the Triton-insoluble fraction (cytoskeletal proteins) were Mr=53,000 polypeptide (vimentin) and actins (Fig. 1c, d). The density of the Mr =53,000 polypeptide was much the same between B16-F1F and B16-F10F. As seen in the total proteins, the relative decrease of A^X actin with respect to β- plus γ-actin in the cytoskeletal fraction was evident in F10F (Fig. 1d) when compared to B16-F1F, in which the ratio of A^X actin:β-actin:γ-actin was approximately 1:1:1. In addition to the A^X actin, the peptides indicated by the arrows in Fig. 1a, b also appeared to differ reproducibly in expression between B16-F1F and F10F cells.

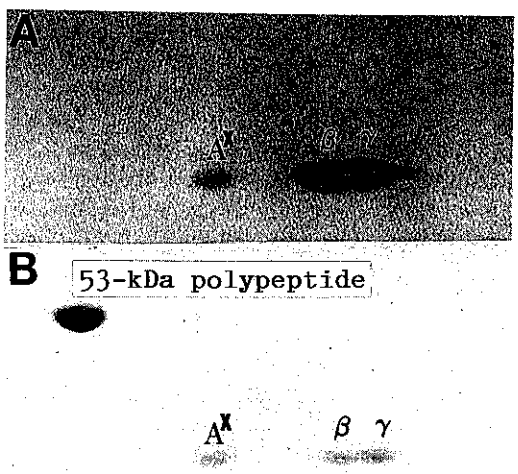

Fig. 2. Reaction of actins in B16-F1F cells with monoclonal anti-actin antibody. Triton-insoluble polypeptides of B16-F1F cells were separated by two-dimensional gel electrophoresis. The section of actins (Mr, 40,000–50,000; pI, 4.5–6.0) was electrophoretically blotted onto nitrocellulose and the blot was stained, as described under "Materials and Methods." While three spots (Mr, 43,000; pI, 5.2–5.3) reacted with anti-actin antibody, a large polypeptide spot (Mr, 53,000; pI, 5.1) of vimentin was not stained. A, Immunologically stained actin spots blotted onto nitrocellulose. B, Coomassie Blue-stained two-dimensional gel corresponding to the section of actins after blotting onto a nitrocellulose filter.

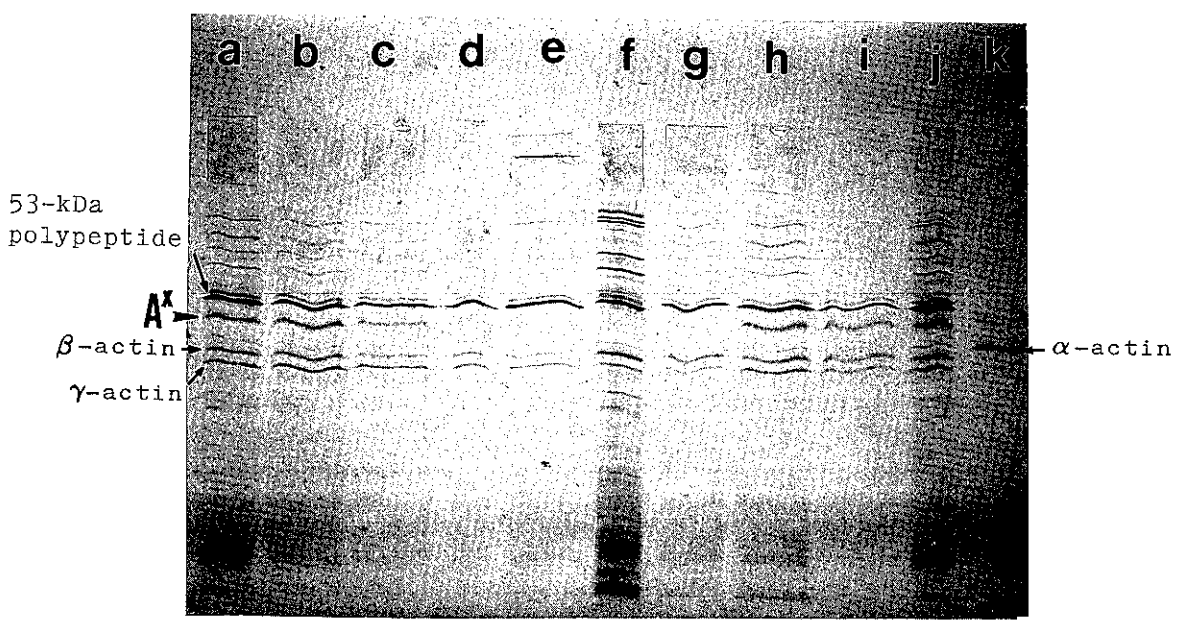

Fig. 3. Horizontal isoelectric focusing of cytoskeletal polypeptides from B16 melanoma lines of different selection cycles. a, B16-F1F; b, B16-F3F; c, B16-F4F; d, B16-F7F; e, B16-F10F; f, B16-BL6; g, B16-F10; h, B16-F1 (continuously cultured for 5 months); i, B16-F1 (cultured 27 days after recovery from liquid nitrogen); j, parent B16 melanoma; k, skeletal muscle α-actin of rabbit, as an authentic sample. The regions of sample application are the areas with some distortion.

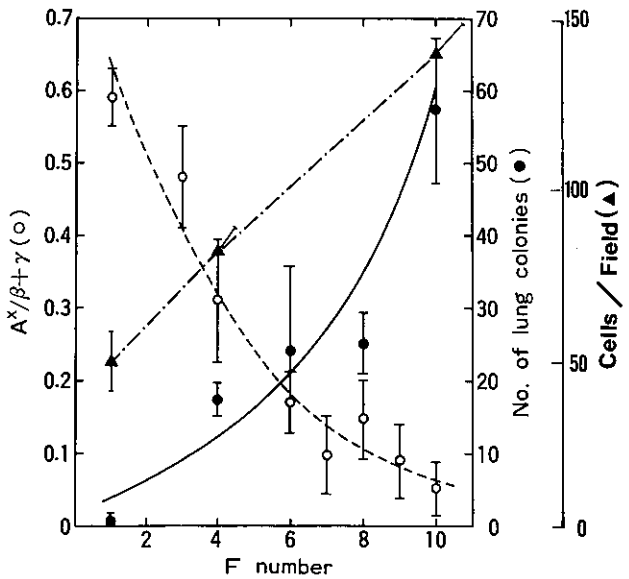

Fig. 4. Inverse relation of alteration in A^X actin expression to metastatic ability and *in vitro* invasiveness of B16 melanoma after various numbers of *in vitro* and *in vivo* selection cycles. The abscissa shows the number of *in vitro* and *in vivo* selections for high-metastatic cells. Open circles show the ratio of A^X actin to β- plus γ-actin calculated from the density of each actin band in horizontal isoelectric focusing gel stained with Coomassie Blue. Each point indicates the mean of triplicate determinations (three gels). Bars, SE. Lung colonization was assessed as described in "Materials and Methods." Mice (13 weeks old) were killed 21 days after iv administration of B16 melanoma cells and the lungs were placed in Bouin's fixative. The number of metastatic foci on the lung surface was counted. Each point (closed circle) shows the mean of 8–9 mice. Bars, SE. The significance of differences of lung colonies was evaluated by using the Mann-Whitney test. *P* values for B16-F1F vs. B16-F4F: <0.01; B16-F8F vs. B16-F10F: <0.05. Triangles show the number of cells that penetrated through matrigel-coated filters per field ($\times 200$). Each point indicates the mean of triplicate samples. Bars, SE. The significance of differences in the number of cells per field was evaluated by using Student's *t* test. *P* values for B16-F1F vs. B16-F4F: <0.05; B16-F4F vs. B16-F10F: <0.01.

Horizontal isoelectric focusing of several B16 melanoma cell lines with different numbers of *in vitro* and *in vivo* selections An illustrative flat isoelectric focusing gel stained with Coomassie Blue revealed differences in the amount of A^X actin among several B16 melanoma cell lines. Each band of actin isoforms and Mr=53,000 polypeptide was identified by comparison with the patterns seen in two-dimensional gels and the localization of authentic α-actin from rabbit skeletal muscle, and from the pH gradient of the gel (Fig. 3).

Table I. A^X Actin Expression in Subclones of B16-F1F and B16-F10F

$A^X/\beta+\gamma$[a]	Number of subclones/total number of subclones	
	F1F	F10F
<0.2	0/18	22/22
0.4–0.6	18/18	0/22

a) The ratio was densitometrically determined on Coomassie Blue-stained gels as described in "Materials and Methods."

In agreement with our previous findings[7] obtained using two-dimensional gel electrophoresis, low-metastatic parent B16 melanoma (j) and two B16-F1 (h, i: these two differ in the period of culture *in vitro*) expressed A^X actin in an amount comparable to β-actin or γ-actin, while A^X actin was not detected in the high-metastatic B16-F10 (g) isolated by Fidler,[8] or in B16-BL6 (f). The expression of A^X actin in the B16 melanoma cell line isolated in the Fujisawa Pharmaceutical Company decreased concomitantly with increase in the number of selections (F number) of the cell lines (a, B16-F1F; b, B16-F3F; c, B16-F4F; d, B16-F7F; e, B16-F10F). For a quantitative assessment of the decrease in expression of A^X actin, the ratio of A^X actin to β- plus γ-actin was densitometrically determined, using three gels (Fig. 4).

Expression of A^X actin in subclones of B16-F1F and B16-F10F To know whether B16-F10F is a mixture of A^X producing and non-producing cells, we examined 22 clones derived from B16-F10F and found no clone expressing A^X actin to the same extent seen in the B16-F1F or F1 (Fidler's) cells (Table I). The expression of A^X actin was homogenously low and the ratio of A^X to β- plus γ-actin in the clones was less than 0.2 (average, 0.1). On the other hand, we have detected no subclone of B16-F1F or F1 (Fidler's) expressing as low an amount of A^X actin as B16-F10F. The average ratio of A^X to β- plus γ-actin in the subclones of F1F was about 0.5.

Depression of A^X actin with increase in metastatic ability and *in vitro* invasiveness of B16 melanoma cell lines As shown in Fig. 4, the ratio of A^X actin to β- plus γ-actin clearly decreased, roughly from 0.5 (B16-F1F) to 0.1 (B16-F10F). On the other hand, Fig. 4 also shows that the metastatic potential of B16 melanoma cell lines was augmented with increase in the F number. The difference between B16-F1F and B16-F4F or between B16-F8F and B16-F10F was statistically significant, though there was no significant difference among intermediate cell lines (F4F,F6F,F8F). Although the absolute average number of lung colonies differed from experiment to experiment, evidence that the lung metastasis increased with increase in the F number was reproducibly ob-

tained. In a different experiment using female C57BL/6 mice of different ages (9 weeks), the numbers of lung colonies produced by iv inoculation with 1×10^5 cells were as follows: B16-F1F, 11; B16-F4F, 37; B16-F10F, >150.

As shown in Fig. 4, the ability of the cell lines to penetrate the reconstituted basement membrane matrigel increased with the increase in F number. B16-F10F showed the highest migration (about 150 cells per field), whereas B16-F1F showed the lowest (about 50 cells per field). The number of cells penetrating through the matrigel coated filter was significantly different among B16-F1F, B16-F4F, and B16-F10F cells.

Synthesis and decay of A^X actin in F1F and F10F cells Synthesis and decay of A^X actin in B16-F1F and B16-F10F were examined to observe mechanisms related to different expressions of A^X actin between those two cell lines. As shown in Fig. 5A, the synthesis of A^X actin significantly higher in B16-F1F than in B16-F10F cells, while the rate of synthesis of β- plus γ-actin was much the same between the two cell lines. The decay curves (Fig. 5B) of A^X actin in B16-F1F and B16-F10F cells were almost in parallel with those of β- plus γ-actin. Thus, A^X actin and β- and γ-actins have much the same half lives (about 35 h) in these cell lines. The difference in the constitutive amount of A^X actin between B16-F1F and B16-F10F cells is attributed to different rates of synthesis of A^X actin.

Organization of cytoskeletal actin in B16-F1F and B16-F10F cells The organization of cytoskeletal actin in B16-F1F and B16-F10F cells growing on glass was examined by direct immunofluorescence with rhodamine-conjugated phalloidin. An illustration of the stained cells is given in Fig. 6. In the B16-F1F cells, actin stress fibers were observed in a large population of the cells while they were less prominent in the B16-F10F cells. In the B16-

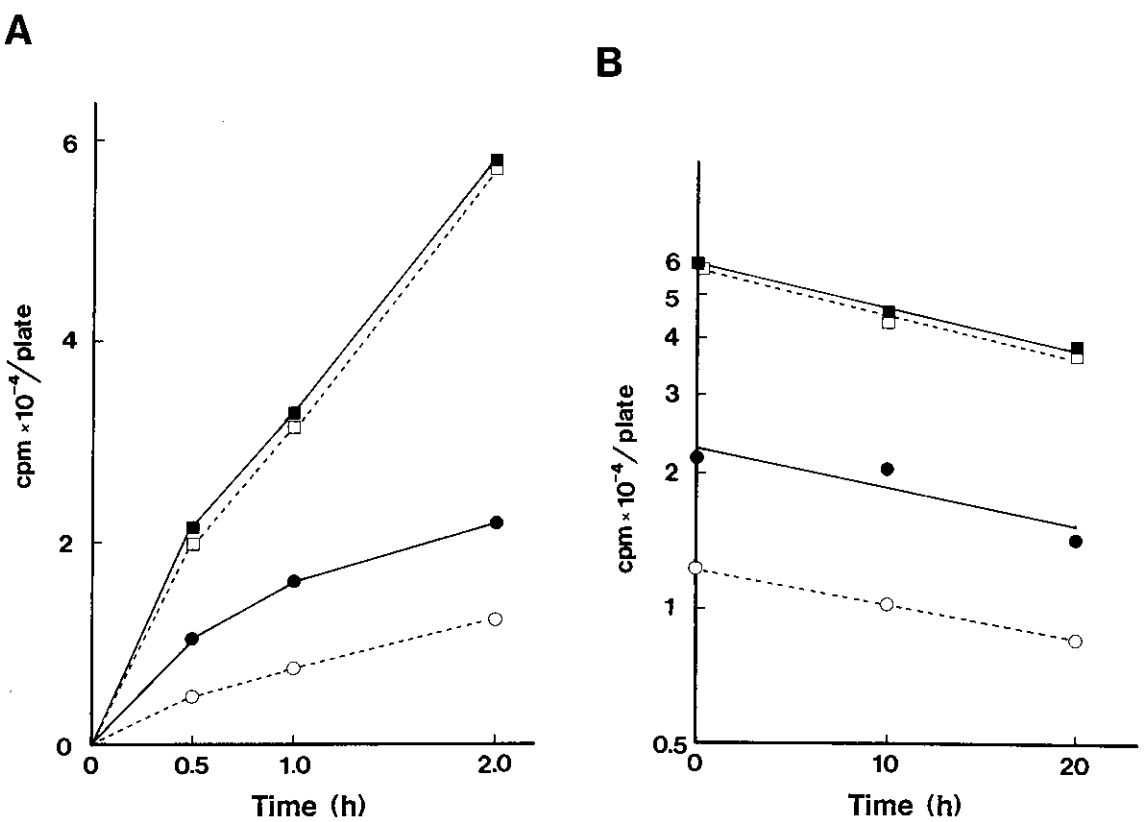

Fig. 5. A, Synthesis of actins in B16-F1F and B16-F10F. Cells were incubated with [^{35}S]methionine until the indicated times, and then total cells extracted with acetone were lysed with O'Farrell's buffer[9] for two-dimensional electrophoresis. Each point indicates the radioactivity of the actin spot (A^X actin, β- plus γ-actin) excised from the gels. B, Determination of the half life of actins. Cultures labeled with [^{35}S]methionine for 2 h were chased with cold methionine up to the indicated times. The cells were lysed and electrophoresed, and the radioactivity was counted for actin spots, as described above. ■: β- plus γ-actin of B16-F1F; □: β- plus γ-actin of B16-F10F; ●: A^X actin of B16-F1F; ○: A^X actin of B16-F10F.

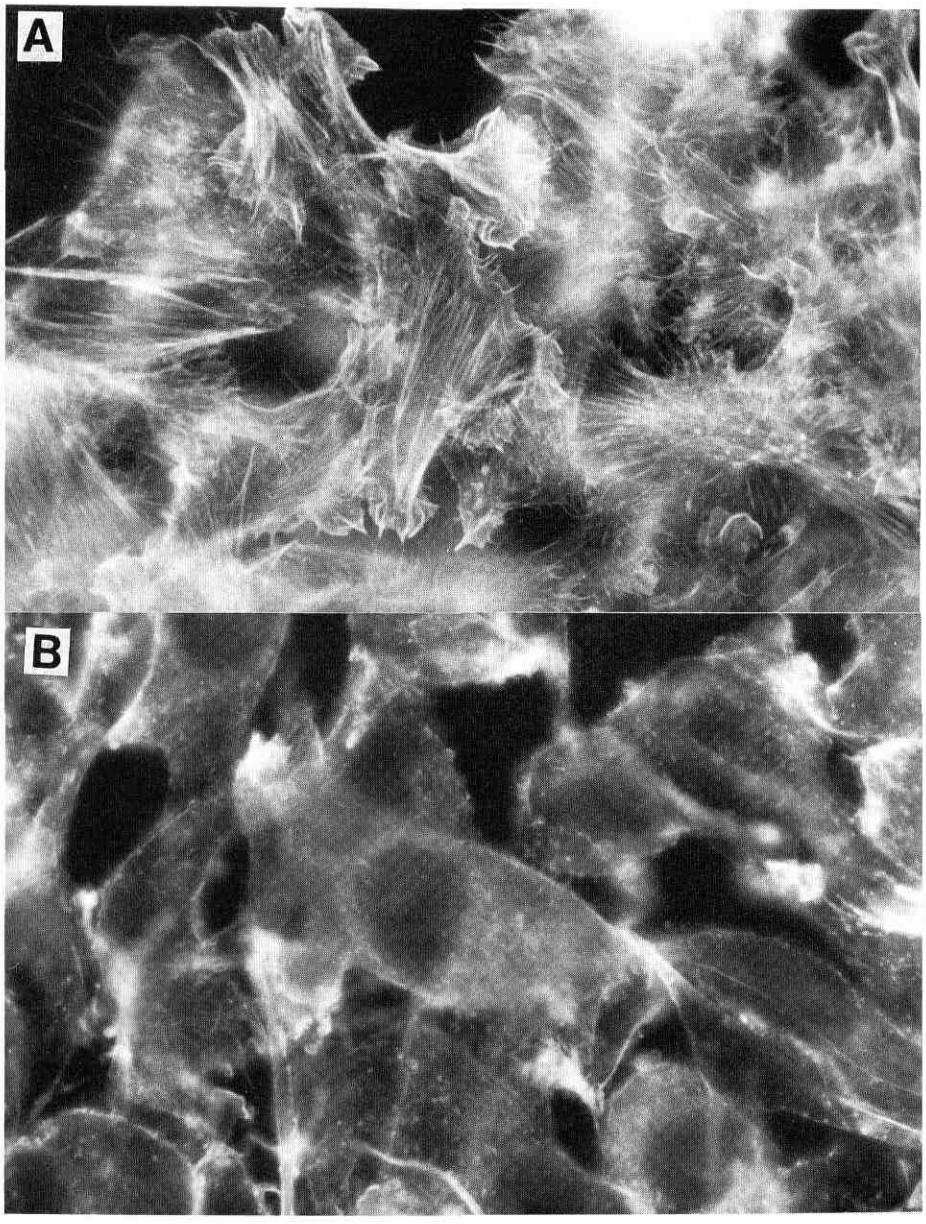

Fig. 6. Cells stained with rhodamine-phalloidin (×200). A, B16-F1F; B, B16-F10F.

F10F cells, we observed thinner actin filaments and dot spots which looked like fragmented actin cables.

DISCUSSION

The main new finding in this work is that the expression of actin A^X (besides β- and γ-actin) is inversely correlated with the increase of the malignancy, as assessed by lung colony-forming ability and *in vitro* invasiveness, in mouse B16 melanoma cells.

The gradual decrease of A^X actin expression associated with the increased metastatic potential may be due to enrichment during the selection process of cells not producing A^X. Our data shown in Table I, however, suggested that this is not the case. B16-F10F cells (A^X actin/β- plus γ-actin $\fallingdotseq$ 0.1) did not consist of a mixture

of A^X-producing (A^X actin:β-actin:γ-actin=1:1:1) and non-producing (A^X:β-actin:γ-actin=0:1:1) cells.

According to examination of the synthesis and decay of A^X actin protein, differences in the constitutive amount of A^X actin between B16-F1F and B16-F10F cells were attributed to the rate of synthesis and not to the rate of decay of A^X actin. Hence, the amount of translatable mRNA apparently differs in these cells. In *in vitro* translation, the ratio of A^X actin synthesized to β- and γ-actin was larger (0.6) in the B16-F1F cells than in the B16-F10F cells (0.3) (data not shown). These results are consistent with our previous data on *in vitro* translation by mRNA from B16-F1 and B16-F10,[7] isolated by Fidler: mRNA coding for A^X was identified in B16-F1 but was scarce in B16-F10..

The concomitant decrease of A^X actin with the increase of metastatic ability in B16 melanoma cell lines isolated by Fujisawa Pharmaceutical Co. Ltd. may be due to alteration in the regulating region of the A^X actin gene or in the expression of regulation factors, such as enhancer or repressor, or in the stability of translatable mRNA, etc. Abtecol *et al.*[15] noted that actin in skin fibroblasts from individuals predisposed to dominantly inherited cancers had an increased turnover rate, increase in incorporation of [^{35}S]methionine into actin and a reduced half-life, as compared with their normal counterparts. Thus, the mechanism of the alteration in expression of A^X actin accompanying malignant progression apparently differs from the case of skin fibroblasts predisposed to cancer. According to Goldstein and Leavitt,[16] human osteosarcoma-derived fibroblasts (HOS) expressed β- and γ-actin in a ratio of 2:1, while the cells transformed by the chemical carcinogen MNNG (N-methyl-N′-nitro-N-nitrosoguanidine) synthesized 50% less β-actin, hence giving a ratio of β:γ=1:1. They predicted that one of two functional β-actin genes expressed in HOS cells was inactivated in MNNG-HOS cells by either a regulatory or structural gene mutation. Recently, we cloned A^X actin cDNA,[17] and found that the sequence of A^X actin had one different base in the coding region, and one base exchange, one insertion and four deletion sites in the untranslated region, as compared with β-actin.[18] The one base difference in the coding region corresponded to alteration of the 28th amino acid from arginine of β-actin to leucine of A^X actin, which explained the more acidic pI value of A^X actin protein compared with that of β-actin. These sequence data suggested that the A^X actin gene is a new type similar to but not an allele of the known β-actin gene. Most recently, DePonti-Zilli *et al.*[19] reported that a 40 base pair sequence in the 3′-transcribed but untranslated region of the β-actin gene is involved in the regulation of expression. It remains to be examined whether or not the different structure of the 3′-untranslated region of A^X-actin from that of β-actin is related to the alteration of the A^X actin expression accompanying malignant progression. To search for regions linked to regulation of the expression of the A^X actin gene, cloning of the genomic A^X actin gene is under way.

The polymerizing function of A^X actin *in vivo* seems to be normal, because the ratio of A^X actin to β- plus γ-actin in the cytoskeletal fraction is the same as the ratio in the Triton-soluble fraction. Hence, this A^X actin may function to maintain the structure of the microfilaments. This is consistent with the results of staining experiments with rhodamine-phalloidin, showing that the organization of actin stress fibers was better in B16-F1F than in the B16-F10F line. In the *in vitro* assay system for cell invasiveness, we found that the invasive potential was increased with decrease in the expression of A^X actin (Fig. 4). This is in accord with the report by Terranova *et al.*[20]: invasiveness *in vitro* is greater in B16-F10 and BL6 not expressing A^X actin than in B16-F1 expressing A^X actin. In our preliminary gene transfer experiments, we observed that A^X actin gene transfer induced an increase in actin stress fibers and a decrease in *in vitro* invasiveness (unpublished results). On the contrary, a point-mutated β-actin (at amino acid 244, glycine to aspartate) found by Kakunaga *et al.* in a transformed human fibroblast cell line (HUT-14), has a low polymerization ability compared to normal β- and γ-actin *in vivo*[21, 22] and *in vitro*,[23] and seems to play a role in the disorganization of actin stress fibers. Leavitt *et al.* noted that transfer of the mutant β-actin gene into the benign cell line increased the malignancy and altered organization of the microfilaments.[24, 25] Thus, it may be that both the depression of A^X actin in B16 melanoma and the expression of mutant β-actin in the case of HUT-14 result in alteration of cell stiffness and/or motility and so on, leading to malignant transformation or progression. Alterations in the organization of actin stress fibers and physiological functions of the B16 melanoma cells, including invasiveness and motility, perhaps are caused not only by change in the expression of A^X actin but also by change in other polypeptides. We did note alterations in the expression of several polypeptides accompanying malignant progression (Fig. 1), but their significance remains to be determined.

There are reports of a depression of a third functionally normal actin associated with cell transformation. In passaged chick embryo fibroblast cultures, Witt *et al.*[26] observed a selective reduction in the amount of an α-actin in cells transformed by the Rous sarcoma virus and little or no reduction of β- and γ-actin. Leavitt *et al.*[27] noted that early passage mouse and rat fibroblast strains synthesized the three electrophoretic forms of actin (β-, γ- and α-actin), but mouse and rat cancer cells expressed only the β- and γ-actin. We noted that a third actin,

smooth muscle α-like actin, was expressed in human pigment cells and tissues but not in malignant counterparts (unpublished data). The significance of the presence of multiple actin isoforms and the mechanism of alteration in their expression in certain cells[28-31] is unknown. A^X actin will be useful in studies on the effect of alteration in the organization of actin stress fibers on malignant progression, on the relationship between molecular structure and function of actin and on mechanisms of regulation of expression of the actin gene.

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