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Novel Non-tumorigenic Cell Variants Showing Potentially Different Susceptibility to v-*src*-induced Metastasis

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Two non-tumorigenic variant cells were isolated from UV-irradiated Balb/c 3T3 cells on the basis of their different responsiveness in phorbol ester-induced morphological change (rounding formation). They showed marked differences of lung metastatic potentials after intravenous injections of their v-*src* transformants into nude mice; phorbol ester-resistant variant TR4 cells transformed by v-*src* were hypermetastatic, whereas v-*src* transformants of phorbol ester-sensitive variant TR5 cells were not metastatic at all. These different metastatic responses were not observed in v-K-*ras*-induced transformants of the variants. These non-tumorigenic variant cells may pre-acquire the genetic alteration of certain *src*-specific and metastasis-associated factors. This system may be useful for genetic analysis of the induction of metastasis.

Key words: Potentially metastatic variants — Balb/c 3T3 — Phorbol ester — Oncogene

Identification of cellular and genetic factors which regulate metastatic behavior of tumor cells is important for our understanding of tumor biology and for the cure of patients harboring malignant tumors. Previous studies have provided evidence that various oncogenes induce metastatic phenotype of cultured cells.[1-3] However, tumorigenic cells transformed by oncogenes and chemical carcinogens do not always exhibit metastatic properties.[2,4] Thus, metastatic propensity is distinct from tumorigenicity,[5,6] suggesting that cellular genes responsible for metastasis may be different from known oncogenes although they may interact with each other in the process of tumor progression. As a first step towards identification of the cellular and genetic factors that control metastatic behavior of tumor cells, we attempted to isolate non-tumorigenic but potentially metastatic variants from Balb/c 3T3 cells. In the present report, we describe the oncogene-induced metastatic responsiveness of two novel non-transformed Balb/c 3T3 variant cell lines originally isolated from parental Balb/c 3T3 A31-1-1 cells on the basis of their different morphological responses to phorbol ester tumor promoters.

MATERIALS AND METHODS

Materials Balb/c female *nu/nu* mice were obtained from Clea Japan. Eagle's minimum essential medium was from Nissui (Tokyo). Fetal bovine serum was from Bocknek Lab. 12-O-Tetradecanoylphorbol-13-acetate (TPA) was purchased from Sigma. Antiserum against v-*src* (Rous sarcoma virus-specific tumor-bearing rabbit serum) was from Transformation Res. Inc.

Cell cultures Balb/c 3T3 A31-1-1 and the variant cells were maintained as previously described.[7]

Isolation of morphologically TPA-resistant and -sensitive cell variants Phorbol ester tumor promoters, represented by TPA, have been shown to rapidly and transiently induce morphological change (so-called rounding formation) of Balb/c 3T3 cells.[8,9] To obtain TPA-resistant and -sensitive variants, approximately 5×10^5 cells, seeded the day before in 100-mm dishes, were irradiated with ultraviolet (UV) (5 J/m^2) and cultured for two days to allow the cells to grow. Then, these cells were treated with TPA (100 ng/ml). After 1–2 h, morphologically TPA-unresponsive cells were marked under the phase-contrast microscope, and then surrounding TPA-responsive cells were removed with micro-pipettes. After two weeks, the marked colonies were isolated using cloning rings and re-cloned. Similarly, morphologically TPA-sensitive variants were obtained by cloning the cells with delayed recovery from TPA-induced morphological rounding.

Cell transformation by oncogenic viruses and experimental metastasis Cells were infected with a recombinant retrovirus containing v-*src* oncogene and/or the neomycin-resistance gene, and with Ki-MSV containing v-K-*ras*. Each transformed clone (which was not releasing virus) was isolated from an independent focus from different infections. Exponentially growing cell cultures were washed with Ca^{2+}, Mg^{2+}-free phosphate-buffered saline, trypsinized, and resuspended in Hanks' balanced salt solution. Aliquots of 2×10^5 cells in 0.15 ml were each injected into the lateral tail vein of 4-week-old Balb/

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c female *nu/nu* mice for the experimental metastasis assay.[10, 11] Twenty-one days later, mice were killed by ether anesthesia. The lungs were removed and metastases counted under a dissecting microscope. Tumorigenicity of the cells was evaluated by monitoring tumor formation after subcutaneous injection of 2×10^5 cells. Latency was scored as the day at which a 2-mm tumor was detectable.

***In vitro* kinase assay of pp60$^{v\text{-}src}$** The protein kinase assay was carried out according to Inoue *et al.*[12] Briefly, cell lysate was prepared by incubation of cells with 1.0 ml of RIPA buffer (150 m*M* NaCl, 1% sodium deoxyholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 10 m*M* Tris hydrochloride, pH 7.2) containing 100 kallikrein inactivator units (KIU) of Trasylol per ml, 20 m*M* NaF and 10 m*M* EDTA, and centrifuged at 10,000*g* for 3 min at 4°C. For immune precipitation, the supernatant containing 100 μg protein was incubated for 1 h at 4°C with 5 μl of *src*-antiserum. The immune precipitates were suspended in 25 μl of kinase buffer (20 m*M* Tris-HCl, pH 7.4, 10 m*M* $MnCl_2$), incubated with 5 μCi of [γ-^{32}P]ATP for 10 min, and analyzed by SDS-polyacrylamide gel electrophoresis.

RESULTS AND DISCUSSION

Two stable variant clones which exhibit different responses in TPA-induced morphological change (rounding formation) were finally isolated from 2×10^7 UV-irradiated Balb/c 3T3 cells (Fig. 1). One variant, TR4, was resistant to TPA-induced morphological rounding. The other variant, TR5, had a relatively long process and was sensitive to TPA-induced rounding, which was similar to 1-1 cells. However, the recovery of TR5 cells from TPA-induced rounding was slower than that of 1-1 cells. Although the mechanisms of phorbol ester-induced morphological rounding formation are unknown, we have found no difference in the number of phorbol ester receptors or the activity of protein kinase C among these cells.[13]

In order to investigate a possible relationship between the morphological responsiveness and metastatic behavior of these variant cells, we assessed the ability of the variant cells transformed by v-*src* and v-*ras* oncogenes to form experimental metastases. As shown in Table I, the variant cells and their neo-resistants did not produce any tumor upon subcutaneous injection into nude mice, indicating that these variants are themselves non-tumorigenic. Parent 1-1 cells transformed by v-*src* formed only a few metastatic nodules in the lungs. On the other hand, v-*src* transformants of TR4 cells extensively metastasized throughout the lungs of nude mice. The average number of lung nodules formed by the transformed TR4 cells was 50–100 times more than that of transformed 1-1 cells (Table I). In contrast, no metastases were detected in the case of v-*src* transformants of TR5 variant cells, indicating that the TR5 cells were resistant to v-*src* induced metastasis. The differences of v-*src* induced metastatic potentials among these cells were not due to different tumorigenicity because the frequency and latency of tumorigenicity of the transformed cells were similar among them (Table I). In contrast to v-*src*, v-K-*ras* transformants of the variants

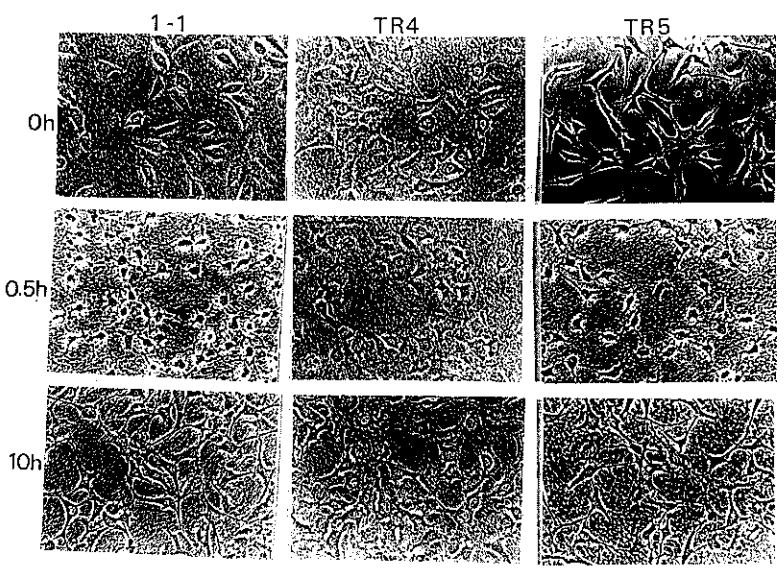

Fig. 1. The TPA-induced morphological changes of Balb/c 3T3 cell variants (rounding formation). Parent (1-1) and variant (TR 4 and 5) cells, which were cultured on plastic dishes, were treated with 100 ng/ml of TPA. In non-treated cultures, 1-1 and TR4 cells were not distinguished from each other. TR5 cells had relatively long processes (arrows). Note that parent and TR5 cells became morphologically round leaving some long processes (arrowheads) within 0.5 h after TPA treatment, whereas no pronounced change was observed on TPA-treated TR4 cells.

Table I. Metastasis Formation by Balb/c 3T3 Variants Transformed by v-*src* and v-K-*ras* Oncogenes

Cell line	Transforming gene	Experimental metastasis		Tumorigenicity	
		Frequency	Lung nodules[a]	Frequency	Latency (days)
1-1	—	0/4	0	0/6	>50
neo	neo	0/4	0	0/4	>50
src-1	v-*src*	4/4	3.2±1.0	5/5	6–7
src-2	v-*src*	4/4	5.2±2.3	5/5	6–7
src-3	v-*src*	3/4	2.6±1.8	5/5	6–7
TR4	—	0/4	0	0/4	>50
neo	neo	0/4	0	0/4	>50
src-1[b]	v-*src*	4/4	187.1±22.6	4/4	7–9
src-2[b]	v-*src*	4/4	211.4±34.7	4/4	7–9
src-3[b]	v-*src*	4/4	167.7±18.9	4/4	7–8
src-4[b]	v-*src*	4/4	223.1±36.7	4/4	7–8
TR5	—	0/4	0	0/4	>50
neo	neo	0/5	0	0/4	>50
src-1	v-*src*	0/5	0	4/4	8–10
	v-*src*	0/3[c]	0	—	—
src-2	v-*src*	0/4	0	4/4	8–11
	v-*src*	0/3[c]	0	—	—
src-3	v-*src*	0/4	0	4/4	8–10
	v-*src*	0/3[c]	0	—	—
1-1					
ras-1	v-*ras*	4/4	32.1±6.7	4/4	5–6
ras-2	v-*ras*	4/4	29.7±5.1	4/4	5–6
ras-3	v-*ras*	4/4	36.2±4.4	4/4	5–6
TR4					
ras-1	v-*ras*	4/4	38.1±7.6	4/4	5–7
ras-2	v-*ras*	4/4	42.8±6.2	4/4	5–7
ras-3	v-*ras*	4/4	31.6±4.3	4/4	5–6
TR5					
ras-1	v-*ras*	4/4	28.3±6.2	4/4	6–8
ras-2	v-*ras*	4/4	21.9±4.5	4/4	5–7
ras-3	v-*ras*	4/4	27.5±7.9	4/4	6–8

Metastatic potential of transformed clones was determined by experimental metastasis assay following intravenous injection of 2×10^5 cells (see "Materials and Methods").
a) Number of nodules per lung (±SD).
b) In the case of v-*src*-transformed TR4 clones, mice were killed 16–18 days later because they could not survive for 21 days, due to extensive lung metastases.
c) Lung metastasis was assayed at 5 weeks after intravenous injection.

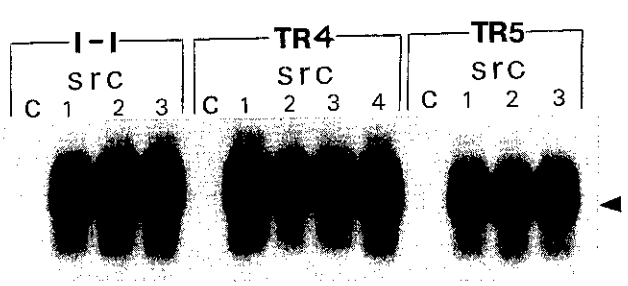

Fig. 2. *In vitro* protein kinase assay of $pp60^{v\text{-}src}$ in v-*src*-transformed variant clones. The assay was performed as described in "Materials and Methods." Phosphorylated IgG heavy chain is indicated by an arrowhead. C, non v-*src*-infected cells; src 1–4, v-*src* transformed clones (the clones correspond to those in Table I).

Table II. Rapid Acquisition of an Experimental Metastatic Phenotype after Infection of Balb/c 3T3 Variant Cells with Viruses Containing v-*src* and v-K-*ras* Oncogenes

Cell line	Oncogene	Transformants per 10^6 cells	Experimental metastasis		Tumorigenicity	
			Frequency	Lung nodules	Frequency	Latency (days)
1-1	—		0/4	0	0/7	>50
1-1	v-*src*	7.4×10^4	4/5	3.8 ± 1.6	4/4	6–7
TR4	—		0/4	0	0/8	>50
TR4	v-*src*	6.2×10^4	4/4	127.2 ± 14.3	4/4	8–10
TR5	—		0/4	0	0/6	>50
TR5	v-*src*	5.5×10^4	0/4	0	4/4	8–11
1-1	v-*ras*	1.2×10^5	4/4	35.2 ± 6.7	4/4	5–6
TR4	v-*ras*	0.9×10^5	4/4	42.1 ± 8.6	4/4	5–7
TR5	v-*ras*	0.8×10^5	4/4	31.1 ± 4.4	4/4	6–8

Approximately 1×10^6 cells, seeded the day before in 100-mm dishes, were infected with viruses. The following day the cultures were split, and 2 days later the infected cultures were trypsinized. Then, the unselected cells (1×10^6) were injected into the tail veins of nude mice. The number of transformed cells was calculated by focus assay on sister dishes that were held for 2–3 weeks before being scored. As the doubling times of 1-1, TR4, TR5 at the exponential growth phase were about 17.0, 17.5, 18.5 h, respectively, population increase of the cultures was approximately 3.5–4.0 fold between infection and injection.

showed metastatic potential similar to that of v-K-*ras*-transformed 1-1 cells (Table I).

As the extent of expression of oncogenes has been reported to affect phenotypic expression of transformation[14] and metastatic potential,[15] we analyzed the protein kinase activities of $pp60^{v\text{-}src}$ of each v-*src* transformed clone. As shown in Fig. 2, the protein kinase activity of each clone was not correlated with the metastatic potential (compare Table I). For example, the kinase activities of TR4 src-2 and -3 clones were almost the same as or somewhat lower than those of v-*src*-transformed 1-1 and TR5 clones, in spite of marked differences of their metastatic potentials.

There is a possibility that the different metastatic potential of variant cells transformed by v-*src* may be due to genetic instability as a result of oncogenic transformation and the subsequent cloning process.[16–18] To test this, one million unselected cells, which were infected with oncogenic viruses, were intravenously injected (Table II). Between infection and injection approximately 4 population doublings of the cultures occurred. Experiments shown in Table II confirmed the observation that the v-*src*-transformed TR4 cells were hypermetastatic compared with those of 1-1 and TR5 cells (Table I).

In the present study, we have isolated metastatically variant cells which are non-tumorigenic themselves but show markedly different metastatic potentials upon transformation induced by v-*src* oncogene. To our knowledge, this is the first case in which non-tumorigenic but potentially metastatic variant cells have been clonally isolated. The difference in metastatic potentials was seen by oncogenic transformation with v-*src* but not v-K-*ras* gene. This seems to suggest that the potentially different susceptibility of the variant cells to metastasis may be specific for v-*src* oncogene, although we have not yet tested many other oncogenes. We found no correlation between the metastatic potential and $pp60^{v\text{-}src}$ kinase activity of v-*src* transformed clones, suggesting that the different metastatic potentials might not be due to different expression levels of $pp60^{v\text{-}src}$ but might reflect the intrinsic difference of other cellular properties. Rapid acquisition of metastatic phenotype of TR4 cells after transformation by v-*src* suggests that this variant may pre-acquire the genetic alteration of a certain *src*-specific and metastasis-associated factor which is presumably induced by UV-irradiation.

Since the variant cells have been isolated on the basis of their responsiveness in TPA-induced morphological change, it is likely that the determinant of their metastatic susceptibility may be closely related to that of their responsiveness to phorbol esters. However, the isolation and identification of cellular factors which regulate responsiveness to phorbol esters and oncogene-induced metastatic behavior of these variants remains as a subject for further study. We observed in preliminary cell fusion experiments that the TPA-resistant and hypermetastatic

natures of TR4 cells were recessive. Therefore, it is expected that metastasis-related genes may be isolated and identified by the transfection of normal human and rodent DNA molecules into the variant cells and by subsequent selection for morphological and metastatic responsiveness.

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