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MALIGNANT PROGRESSION OF A TRANSFORMED RAT CELL LINE BY TRANSFER OF THE V-FOS ONCOGENE

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SUMMARY: Transfer of the v-fos oncogene into a rat cell line transformed by Rous sarcoma virus increased both the spontaneous and experimental lung-metastasis. Metastatic ability of each v-fos transferred cell line was dependent on both the manner of integration and transcriptional amount of the v-fos oncogene, but did not correlate with the growth rate in vivo. Expression of the src, myc or ras genes were not altered by transfer of the v-fos gene, except that the myc expression was enhanced in the cell line, which acquired augmentation of growth rate in vivo but not metastatic potential to the lung. Cells of the metastatic lung nodules of each cell line also possessed exogenous fos DNA and the transcripts. These results suggest that v-fos oncogene functions in the transfected cells and causes malignant progression.

Recent developments of molecular and cellular biology revealed that some oncogenes play important roles in the cellular growth and differentiation as well as in transformation (1-5). The molecular and cellular mechanisms related metastasis are not well understood. The possibility of involvement of the fos oncogene in acquisition of metastatic potential has been suggested by two independent groups (6,7). In the present study, we investigated biochemical and/or biological characteristics of the v-fos transferred cell lines, and directed attention to augmentation of the metastatic potential through the gene transfer.

MATERIALS AND METHODS

v-fos gene transfection

A transformed rat fibroblast cell line, SR-3Y1-2, which was established by infection of a normal rat fibroblast cell line, 3Y1-B clone 1-6 (8), with Schmidt-Ruppin D strain Rous sarcoma virus (unpublished data, Mitsudomi and Kimura), was used as the recipient of the cloned v-fos gene, pFBJ-2 (9). pFBJ-2 was co-transfected with pSV2-neo (10) DNA by calcium phosphate precip-

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itation, as described (7). Five independent colonies resistant to Geneticin (G418, Gibco) were isolated and designated as fos-SR-3Y1-201 ~ 205. Cells of the remaining colonies were combined and designated as fos-SR-3Y1-200. All the cell lines were cultured in Dulbecco's modified Eagle medium supplemented with 10 % fetal bovine serum in a humidified atmosphere of 10 % CO_2 and 90 % air.

Spontaneous and experimental lung metastasis in rats

Semi-confluent cells of each cell line were harvested by trypsinization and resuspended in Ca^{2+}, Mg^{2+} free Hanks' balanced salt solution at the density of 2 x 10^5 or 2 x 10^6 viable cells per ml. Viability of each cell line, determined by trypan blue dye exclusion test, exceeded 80 %. Spontaneous lung metastasis was examined, as described (7). Growth of the i.m. inoculated cells was monitored by measuring size of the formed tumor, weekly until the hosts died. In the assay of experimental lung metastasis, 10^5 viable cells were injected into the tail vein of F344 rats. These rats were killed 4 weeks after the injection, and lungs and other organs were examined for metastases. From the metastatic lung nodules, several second generation lines resistant to Geneticin (400 μg/ml) were established. These cell lines were designated by adding -Fl to their parent cell lines.

Southern and Northern blot analysis

Southern and Northern blot analysis in genomic DNA and poly(A^+)RNA of each cell line was performed as described (7), using three ^{32}p-labeled fos-specific DNA probes (2 x 10^8cpm/μg), Bgl II fragment of 5' region, Pst I fragment of middle region, or Sst I-Bgl II fragment of 3' region of v-fos gene in pFBJ-2 DNA.

RESULTS AND DISCUSSION

Southern blot analysis of v-fos transfected cell lines

Fig. 1A shows the Southern blot analysis of the genomic DNA digested with Hind III of the fos transfected cell lines, using the Bgl II fragment of the 5' v-fos region in pFBJ-2 as a probe. All of the cell lines have a common 9.6 kbp DNA fragment which contains the cellular fos gene. fos-SR-3Y1-202, 203, 205, and 200 have one extra 8.2 kbp DNA fragment, while 201 and 204 have two bands of 17 kbp and 26 kbp and one band of 6.7 kbp, respectively. fos-SR-3Y1-200, mixed population, has mainly the 8.2 kbp DNA fragment, thereby indicating that the integration of pFBJ-2 in SR-3Y1-2 cells occurred mostly at a specific site on genomic DNA. The extra bands were also hybridized with middle and 3' region of fos-specific region in pFBJ-2 (data not shown), suggesting that the extra Hind III fragments contained almost the entire region of the v-fos gene. Second generations of the fos-transferred SR-3Y1-2 cells carried the exogenous fos gene, and some showed a rearrangement of this gene. Results for fos-SR-3Y1-200-Fls are shown representatively in Fig. 1A.

Spontaneous and experimental lung metastasis in rats

The spontaneous and experimental lung metastasis of the fos-transferred cell lines are shown in Table 1. The results of spontaneous metastasis were

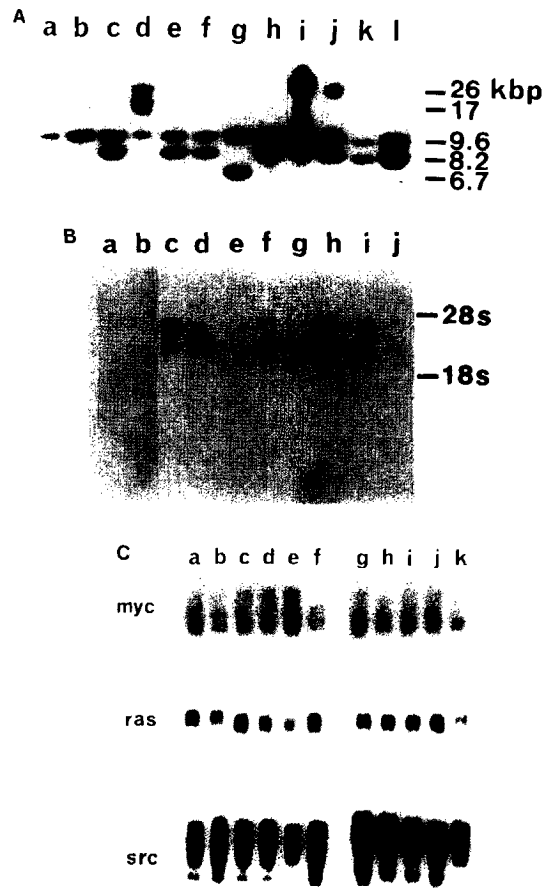

Figure 1. Southern and Northern blot analysis of the fos-SR-3Y1 cell lines: A, Southern blot analysis for fos oncogene in fos-SR-3Y1 cell lines and their parent cell lines, lane a, 3Y1-B clone 1-6; b, SR-3Y1-2; c, fos-SR-3Y1-200; d, 201; e, 202; f, 203; g, 204; h, 205; i, 202-F1-2; j, 202-F1-3; k, 202-F1-1s; l, 202-F1-2s. B, Northern blot analysis for fos oncogene, lane a, 3Y1-B clone 1-6; b, SR-3Y1-2; c, fos-SR-3Y1-201; d, 202; e, 203; f, 204; g, 205; h, 202-F1-3; i, 202-F1-1s; j, 205-F1-3. C, Northern blot analysis for myc, ras, and src oncogene, lane a, SR-3Y1-2; b, fos-SR-3Y1-201; c, 202; d, 203; e, 204; f, 205; g, 202-F1-1; h, 202-F1-2; i, 202-F1-3; j, 202-F1-1s; k, 202-F1-2.

reproducible, when compared to previous reports (7). All the cell lines examined had tumorigenecity, while the number of the metastatic lung nodules varied among the cell lines. Survival of rats inoculated i.m. with fos-transferred cells was much the same or shorter than those of the rats inoculated with control cell lines. Control cell lines, SR-3Y1-2 and neo-SR-3Y1-200, produced 10-50 metastatic nodules in the lungs, spontaneously and experimentally. On the other hand, the three fos-transferred cell lines (fos-SR-3Y1-202, 203, 205) produced several to more than ten times larger

Table I. Spontaneous and experimental lung metastases of fos-transfected SR-3Y1-2 cells

Cell line	Experiment[1]	No. of lung nodules[2] (incidence)	Other metastases	Survival (median)
SR-3Y1-2	A	34.8 ± 23.8 (4/4)	2/4	60
	B	13.0 ± 17.0 (3/4)	0/4	-
	C	7.0 ± 4.6 (4/4)	0/4	-
neo-SR-3Y1-200	A	9.7 ± 10.6 (3/3)	3/3	45.5
	B	47.8 ± 33.2 (5/5)	0/5	-
	C	9.8 ± 5.7 (4/4)	0/4	-
fos-SR-3Y1-201	A	N.D.	-	-
	B	2.3 ± 2.0 (3/5)	0/5	-
	C	3.3 ± 5.6 (3/6)	0/6	-
fos-SR-3Y1-202	A	167 ± 91.1[3] (5/5)	2/3	34
	B	234 ± 44.8[3] (5/5)	0/5	-
	C	150 ± 20.2[3] (4/4)	0/4	-
fos-SR-3Y1-203	A	105 ± 73.5 (5/5)	2/5	49
	B	150 ± 21.3 (5/5)	0/5	-
	C	128 ± 34.1 (4/4)	0/4	-
fos-SR-3Y1-204	A	0.6 ± 0.55[4] (3/5)	1/5	52
	B	4.3 ± 3.3[4] (4/4)	0/4	-
	C	50 ± 16.8[4] (4/4)	0/4	-
fos-SR-3Y1-205	A	76 ± 66.5 (5/5)	0/5	46
	B	208 ± 82.4 (4/4)	0/5	-
	C	157 ± 84.2 (5/5)	0/5	-

Details of procedures are described in materials and methods.
1 Experiment A : Spontaneous lung metastasis, Experiment B,C: Experimental lung metastasis.
2 No. of lung nodules were expressed as mean ± S.D., incidence showed the numbers of rats bearing lung metastases / total rats. Histological examination confirmed the presence of malignant tissue in lung nodules.
3 Statistically significant when compared to control groups (Wilcoxon's test).
4 Statistically insignificant compared to control groups (Wilcoxon's test).

numbers of lung nodules than did the control cell lines; fos-SR-3Y1-200 (mixed population) was also more potent than controls (data not shown). These high-metastatic cell lines had a common fos-related DNA fragment of 8.2 kbp (Fig. 1A). Of the cell lines examined, fos-SR-3Y1-204 and 201, with different sized fos-related DNA fragments, produced a low number of lung nodules, as controls. The increase in the number of metastatic lung nodules by the fos gene transfer was much the same in both spontaneous and experimental metastasis, thereby indicating that enhancement of the metastasis results from an alteration of lodging or growing ability in the lung tissues, or survival in the vessels. It remains to be determined which step of metas-

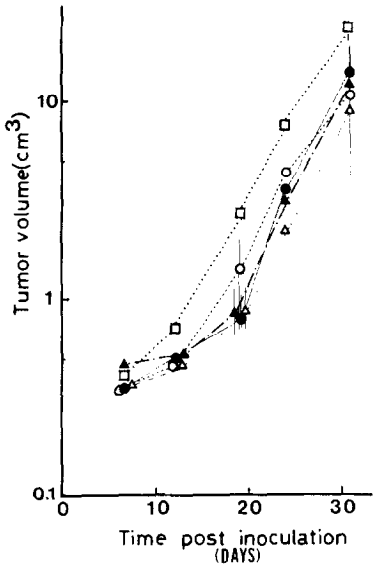

Figure 2. Growth pattern of pSV2-neo- and/or pFBJ-2-transferred SR-3Y1-2 cells in vivo at the inoculation site. Tumor size was measured using a caliper. The growth curve represents the mean tumor volume, which was calculated by $ab^2/2$ (a, long axis; b, short axis). Bars show S.D.. ○ , neo-SR-3Y1-200; ● , fos-SR-3Y1-200; △ , fos-SR-3Y1-202; ▲ , fos-SR-3Y1-203; □ , fos-SR-3Y1-204. P values for neo-SR-3Y1-200 vs fos-SR-3Y1-204 on Day 19 and Day 31:<0.05; for neo-SR-3Y1-200 vs other cell lines except fos-SR-3Y1-204 on Day 9 and Day 31:>0.05 (Wilcoxon's test).

tasis is most enhanced in these cell lines. The rates of tumor growth did not differ among the cell lines, except for fos-SR-3Y1-204 (Fig. 2). The growth of fos-SR-3Y1-204 cells was more rapid than seen in the other cell lines, though it had much the same metastatic potential as the controls. Thus, the increase in metastatic ability to the lung by fos gene transfer did not correlate with the rate of tumor growth. The frequency of spontaneous metastasis to other organs (mostly para-aortic lymph node) or size of the formed nodules was not augmented by the v-fos transfer to SR-3Y1-2 (data not shown).

Expression of fos, src, ras, and myc oncogenes

The size of the transcripts hybridized to the fos-specific probe was about 4.0 kb, which is close to the size of FBJ-MSV proviral DNA cloned as pFBJ-2, except that fos-SR-3Y1-201 expressed the 5 kb transcript (Fig. 1B). The fos gene expression was higher in fos transferred cell lines than in control ones, SR-3Y1-2 and 3Y1-B clone 1-6; fos-SR-3Y1-202, which was the most potent in

metastasis, highly expressed the fos-related transcript, as compared to the other cell lines examined. In fos-SR-3Y1-201, the size of transcript was larger than 4 kb, hence it was probably processed to be translated, in an immature state. In fos-SR-3Y1-204, the expression of the fos gene was larger than that of 203 and 205, as shown in Fig. 1B, the metastatic ability of which was higher than 204. Therefore, the amount of fos-related transcripts does not always correlate with the metastatic potential. As shown in Fig. 1A, however, the integrated size of the fragments containing the fos-specific region differs between 204 and 203 or 205. Thus, both the integration producing 8.2 kbp Hind III fragment and increased transcription are required for the high metastatic potential.

Although it was reported that the fos gene has a transcriptional trans-activation of LTR of Rous sarcoma virus (11), determined by a transient assay (chloramphenicol transacetylase assay), the src expression was not increased in fos-SR-3Y1 cell lines (Fig. 1C), where the LTR is integrated into the chromatin DNA. Immunoprecipitation of src protein also gave similar results (data not shown). The expression of ras and myc genes was also investigated (Fig. 1C), but no remarkable alteration of expression was seen among the controls and fos-transferred cell lines, except that myc expression in fos-SR-3Y1-204, having the largest growth rate in vivo, was augmented. Cell lines of the second generation established from spontaneous and experimental lung metastasis showed much the same level of expression of these oncogenes as did their parent cell lines (Results for fos-SR-3Y1-202-Fls and fos-SR-3Y1-205-F1 are shown representatively in Fig. 1B and C).

It has been suggested that the c-fos gene is involved in the cellular differentiation, proliferation and transformation through its transcriptional trans-activation function (1-3, 11). This is supported by recent findings that the fos protein complex is associated with chromatin and that it has DNA-binding activity in vitro (12, 13). Our results presented here suggest that the v-fos gene also plays a role in progression of the malignant phenotype, such as the acceleration of metastatic potential (fos-SR-

3Y1-202, 203, 205) or of proliferating ability (fos-SR-3Y1-204). Similar results are obtained in experiments of v-fos gene transfer into a ras-transformed rat fibroblast (data not shown). The mechanism of augmentation of malignancy in the v-fos-transferred cell lines remains to be clarified. Further analysis of the biochemical and biological changes induced by v-fos transfer, responsible for the malignant progression, are now under way, with attention being directed to the transcriptional trans-activation function of the v-fos product.

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