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Expression of a *fms*-related oncogene in carcinogen-induced neoplastic epithelial cells

(chemical transformation/growth factor receptors/neoplastic progression)

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ABSTRACT **Following carcinogen exposure *in vitro*, normal rat tracheal epithelial cells are transformed in a multistage process in which the cultured cells become immortal and, ultimately, neoplastic. Five cell lines derived from tumors produced by neoplastically transformed rat tracheal epithelial cells were examined for the expression of 11 cellular oncogenes previously implicated in pulmonary or epithelial carcinogenesis. RNA homologous to *fms* was expressed at a level 5–19 times higher than normal tracheal epithelial cells in three of five of the tumor-derived lines. All three lines expressing high levels of *fms*-related RNA gave rise to invasive tumors of epithelial origin when injected into nude mice. Increased expression of the *fms*-related mRNA was not due to gene amplification, and no gene rearrangement was detected by Southern analyses. RNA blot analysis using a 3′ v-*fms* probe detected a 9.5-kilobase message in the three tumor-derived lines, whereas both normal rat aveolar macrophages and the human choriocarcinoma line BeWo expressed a *fms* transcript of ≈4 kilobases. We conclude from these data that the gene expressed as a 9.5-kilobase transcript in these neoplastic epithelial cells is a member of a *fms*-related gene family but may be distinct from the gene that encodes the macrophage colony-stimulating factor (CSF-1) receptor.**

Almost one-third of the known cellular oncogenes have been implicated in either epithelial or pulmonary carcinogenesis. These include the *ras* (1, 2) and *myc* (1, 3, 4) gene families, *fos* (1), *myb* (5), *raf* (6, 7), *erbB* (8, 9), *abl* (10), and *fms* (1). These otherwise benign cellular genes gain transforming potential as a result of increased or inappropriate expression due to deregulation, gene amplification, or chromosomal translocation, or they may become activated as a result of mutagenic events such as point mutations or gene rearrangement (11). However, the mechanism by which the expression of these cellular oncogenes determines the neoplastic phenotype and the role they play in the progression of a normal cell to a neoplastic variant is not yet fully understood.

In particular, the possible role of the oncogene *fms* in lung malignancies is at present unknown. The oncogene *fms* belongs to the *src*-related tyrosine kinase family; other transmembrane oncogenes of this type include *ros* and *erbB*. These oncogenes are related to the receptors for macrophage colony-stimulating factor (CSF-1) (*fms*) (12), insulin (*ros*) (13), and epidermal growth factor (*erbB*) (14). Whereas v-*ros* and v-*erbB* both exhibit significant truncation of their extracellular domains relative to their cellular cognates, v-*fms* retains the entire extracellular ligand-binding domain of the CSF-1 receptor/c-*fms* molecule (15). Therefore, the expression of a *fms* transcript coding for a macrophage growth factor receptor previously reported in human lung carcinomas (1) leaves open the possibility of contaminating mononuclear cells being responsible for the level of fms mRNA observed.

To study how cellular oncogenes participate in airway epithelial cell carcinogenesis, we have begun to study the expression of cellular oncogenes in primary rat tracheal epithelial (RTE) cells transformed by carcinogens *in vitro*. In this system, normal diploid tracheal epithelial cells can be transformed by carcinogens such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), and the transformants progress with repeated subculture to become immortal and ultimately neoplastic (16), closely paralleling the events that occur during tracheal carcinogenesis *in vivo* (17). Tumors produced by these neoplastic transformants can be explanted into tissue culture, allowing the isolation of tumor-derived cell lines. As end stage transformants, these tumor-derived lines would be expected to express a spectrum of cellular oncogenes that become activated during the neoplastic progression of the transformed cells, and thus may be useful in identifying those cellular oncogenes that play a role in the neoplastic process. In addition, the level of expression of the relevant oncogenes can be quantitated in these lines relative to normal RTE cells.

We report here that three of five tumor-derived epithelial cell lines examined expressed *fms* at a level 5- to 19-fold higher than primary RTE cells. This gene was expressed as a 9.5-kilobase (kb) transcript, whereas normal rat macrophages expressed a 3.8-kb transcript, suggesting that the gene expressed by the transformed RTE cell lines is a *fms*-related gene, which may be distinct from the gene coding for the CSF-1 receptor.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Primary RTE cells were exposed to MNNG or γ irradiation, and individual transformed epithelial foci composed of enhanced growth variants (EGV) were isolated 8 wk after carcinogen exposure; EGV cell lines were clonally isolated by use of cloning cylinders from individual transformed colonies and were subcultured *in vitro* as described (18). Five late-passage EGV cell lines were injected subcutaneously into the backs of nude mice at 2×10^6 cells per inoculum, and the resulting tumors were explanted into tissue culture. EGV T-cell lines were clonally isolated from the tumor explants and cultured in Ham's F-12 medium containing 5% fetal bovine serum, insulin (1 μg/ml), hydrocortisone (0.1 μg/ml), and penicillin/streptomycin/fungizone at 37°C in a humidified atmosphere of 5% CO_2/95% air. The human choriocarcinoma cell line BeWo obtained from American Type Culture Collection was grown in F-12 medium/15% fetal bovine serum and penicillin/streptomycin/fungizone at 37°C in 5% CO_2/95% air. Rat

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Abbreviations: CSF, colony-stimulating factor; RTE cell, rat tracheal epithelial cell; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; EGV, enhanced growth variant(s); EGV-T, EGV tumor-derived cell line; REF, rat embryo fibroblast(s).

aveolar macrophages were harvested as described (19). Differential cell counts performed on the lavage fluid confirmed that 98% of the cells obtained were macrophages.

Cellular DNA and RNA Analysis. High molecular weight DNA was prepared from cultured cells as described (20). DNA was digested to completion at 37°C with the appropriate restriction enzymes and transferred to nitrocellulose after electrophoresis in 0.85% agarose gels by the method of Southern (21). RNA was isolated either by the guanidine thiocyanate method of Chirgwin *et al.* (22) (total RNA) or by cell lysis in cold buffer with 0.5% Nonidet P-40 followed by phenol extraction as described (23) (cytoplasmic RNA). Poly(A)$^+$ RNA was recovered from total cellular RNA after two cycles of chromatography on oligo(dT)-cellulose (24). RNA slot blot analyses were performed with total cytoplasmic RNA in a dilution series (10, 5, 2.5, and 1.25 μg). RNA blot analysis for determination of message size was performed after electrophoresis of poly(A)$^+$ RNA (5 μg) in 0.85% agarose formaldehyde gels and transfer to nitrocellulose (25).

Hybridization and Probes. DNA and RNA filters were baked, prehybridized, and hybridized (26) for 48 hr at 42°C in 40% formamide/3× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate) to ^{32}P-labeled nick-translated probes (specific activity, $>1 \times 10^8$ cpm per μg of DNA) (27). After hybridization, filters were washed with four changes of 1× SSC/0.1% NaDodSO$_4$ at 37°C for 2 hr, dried, and exposed to x-ray film with intensifying screens. Quantitation of RNA expression was achieved by using a Joyce–Loebl Chromoscan-3 densitometer.

Oncogene-specific probes were an *Sst* I/*Cla* I fragment of pSM-FeSV-5′ (5′ v-*fms*) (28) from Charles Sherr and a *Pst* I fragment of the v-*fms* gene (29), pSM-3 (3′ v-*fms*), provided by Rolf Muller.

Tumorigenicity Analysis. EGV cell lines were analyzed for tumorigenic potential as described (18) by injecting 2×10^6 cells s.c. into the backs of nude mice. Tumors were removed after reaching 1 cm in diameter, explanted into tissue culture, and/or fixed in Bouin's fixative, dehydrated, and embedded in paraffin. Histological sections were stained with hematoxylin and eosin.

RESULTS

Tumor-Derived Cell Lines Exhibit Increased *fms* Expression. Five late-passage transformed RTE cell lines, derived from EGV, were injected into nude mice. The resulting tumors were explanted into tissue culture and tumor-derived cell lines (EGV-T) were clonally isolated from the tumor explants (Table 1). Cytoplasmic RNA was isolated from each EGV-T line and slot blot analysis was used to determine the level of expression of 11 cellular oncogenes in these lines relative to normal primary RTE cells. From this analysis, it was determined that N-*myc*, *abl*, *fes*, *erbB*, and *myb* were not detectably expressed by the transformed cells; *myc*, *fos*, *raf*, and Ki-*ras* were expressed at the same level in both transformed and normal cells; and Ha-*ras* was slightly (3-fold) but significantly increased in the transformed cells (unpublished data). One cellular oncogene, *fms*, was expressed at a level 5–19 times greater than that observed in normal RTE cells in three of the cell lines derived from MNNG-transformed tumorigenic cell lines (EGV$_4$-T, EGV$_5$-T, and EGV$_6$-T); EGV$_{10}$-T cells and EGV$_3$-T cells (derived from MNNG-exposed and γ-irradiated RTE cells, respectively) did not express increased levels of *fms*.

Table 1. Tumor-derived epithelial cell lines

Cell line	Transforming agent	Latency period, wk	Tumor histology
EGV$_3$-T	γ irradiation	2	Keratinizing cyst, locally invasive
EGV$_4$-T	MNNG	4	Squamous cell carcinoma, keratinizing, invasive
EGV$_5$-T	MNNG	2	Squamous cell carcinoma, keratinizing, highly invasive
EGV$_6$-T	MNNG	1	Squamous cell carcinoma, keratinizing, highly invasive
EGV$_{10}$-T	MNNG	4	Squamous cell carcinoma, keratinizing, highly invasive

To confirm that EGV-T lines derived from tumor explants that expressed *fms* were of epithelial origin, and not derived from fibroblasts or mononuclear cells that had infiltrated the tumors, these lines were reinjected into nude mice. All three lines expressing high levels of *fms* gave rise to invasive keratinizing squamous cell carcinomas with latency periods of 1–4 wk (see Table 1 and Fig. 1), which were representative of the original tumors from which the EGV-T lines were derived.

Enhanced *fms* Expression Does Not Result from Gene Amplification. To determine whether increased expression of RNA homologous to *fms* occurred as a result of amplification of the c-*fms* gene, high molecular weight DNAs isolated from normal rat embryo fibroblasts and the five EGV-T cell lines were digested with *Hin*dIII and probed for sequences homologous to v-*fms* by Southern analysis. As shown in Fig. 2, both normal rat cells and EGV-T cell lines contained multiple *Hin*dIII restriction fragments, with major bands 6.5, 5.1, 4.3, 2.9, 2.6, and 1.8 kb long. No amplification of these bands was observed in the EGV$_4$-T, EGV$_5$-T, or EGV$_6$-T cells that expressed high levels of *fms* mRNA. In addition, the restriction pattern was the same in all five EGV-T cell lines and was identical to that observed in normal rat embryo fibroblast cellular DNA digested with either *Hin*dIII (Fig. 2) or *Eco*RI (data not shown).

3′ and 5′ v-*fms* Sequences Recognize a 9.5-kb Message in the Transformed Cells. RNA blot analysis was performed on poly(A)$^+$ cytoplasmic RNA isolated from the EGV-T lines. Rat embryo fibroblast (REF) cells instead of normal rat tracheal cells were used as controls for expression of *fms* by the transformants because of the difficulty of obtaining sufficient numbers of tracheal epithelial cells to allow isolation of poly(A)$^+$ RNA (typical yield per trachea, $5–10 \times 10^5$ cells). As shown in Fig. 3,, the *fms*-related mRNA is expressed in EGV$_4$-T, EGV$_5$-T, and EGV$_6$-T as a 9.5-kb transcript, and only those cell lines that were identified by slot blot analysis as expressing high levels of *fms* produced transcripts that hybridized to the 3′ v-*fms* probe. No *fms*-related mRNA was detected in normal REF cells, EGV$_3$-T, or EGV$_{10}$-T. To confirm that this mRNA was indeed homologous to v-*fms*, a 5′ v-*fms* probe was hybridized to EGV-T mRNA. This probe also recognized the 9.5-kb transcript in the EGV$_6$-T cells (Fig. 4) as well as a second smaller transcript of $\approx$4.9 kb, which was present in both REF and EGV$_6$-T cells. This smaller transcript was also present in the other EGV-T lines, including EGV$_3$-T and EGV$_{10}$-T (data not shown).

Because c-*fms*, the cellular homologue to v-*fms*, encodes a product related to the receptor for macrophage CSF-1 (12), it was important to determine whether the 9.5-kb transcript expressed by the transformed rat epithelial cells was the normal message for the rat CSF-1 receptor. The CSF-1 receptor/c-*fms* message has been previously recognized in both human and murine systems as a transcript $\approx$4 kb long (30, 31); therefore, it was also necessary to determine whether the difference in transcript size between the *fms* homologous mRNA detected in rat EGV-T cells and that reported for human and mouse cells was due to species-specific differences. As a source for normal rat CSF-1

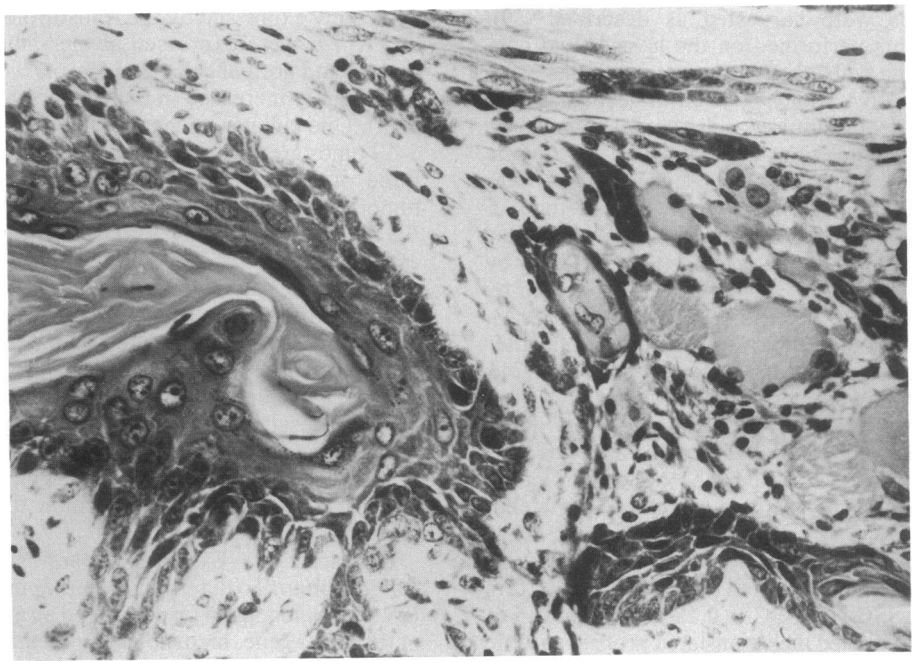

FIG. 1. Histological section representative of invasive squamous cell carcinomas produced by EGV-T cell lines. Paraffin section of EGV_6-T tumor stained with hematoxylin and eosin. (×200.)

receptor message, poly(A)$^+$ transcripts were isolated from total RNA from rat aveolar macrophages and subjected to RNA blot analysis. As shown in Fig. 4, rat macrophages contain v-*fms* homologous transcripts 3.8 kb long as compared to the 9.5-kb mRNA expressed by the transformed EGV_6-T cells. The human choriocarcinoma cell line BeWo also expressed mRNA that hybridized to *fms* of a size similar to the rat macrophages (4.0 kb) (Fig. 4), as has been reported (32). Thus, the presence of a 9.5-kb *fms* homologous message in the transformed epithelial cells cannot be accounted for by species-specific differences in expression of transcripts for the CSF-1 receptor.

DISCUSSION

Experiments presented in this report indicate that of the five tumor-derived cell lines examined, three express increased levels of RNA homologous to the oncogene *fms*. All three cell

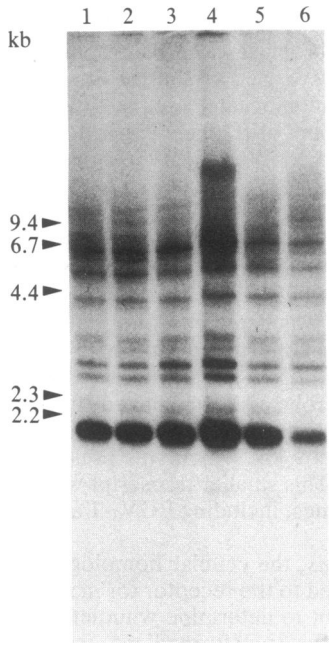

FIG. 2. Southern blot analysis of genomic DNA from normal REF and EGV-T cell lines. DNA samples digested with *Hin*dIII and hybridized to 3′ v-*fms* probe. The amount of DNA loaded per slot was monitored by ethidium bromide staining and by UV illumination of the agarose gel before DNA transfer onto nitrocellulose. After normalization for variations in DNA loading, no amplification of *fms* sequences could be detected in any of the EGV-T lines. Lanes: 1, REF; 2, EGV_3-T; 3, EGV_4-T; 4, EGV_5-T; 5, EGV_6-T; 6, EGV_{10}-T.

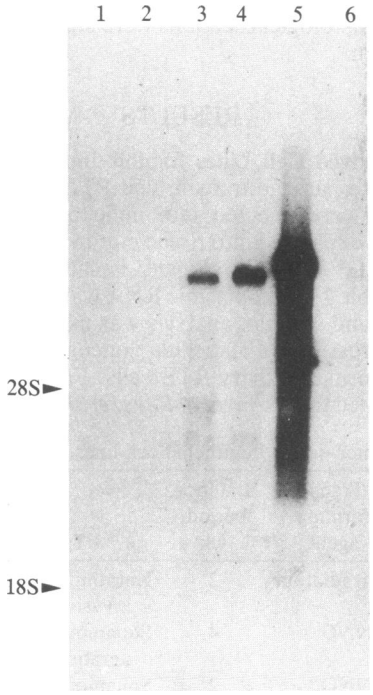

FIG. 3. RNA blot analysis. Samples (5 μg) of poly(A)$^+$ cytoplasmic RNA from REF or EGV-T cell lines were separated by electrophoresis in formaldehyde/agarose gels, transferred to nitrocellulose, and hybridized to 3′ v-*fms* probe. Lanes: 1, REF; 2, EGV_3-T; 3, EGV_4-T; 4, EGV_5-T; 5, EGV_6-T; 6, EGV_{10}-T.

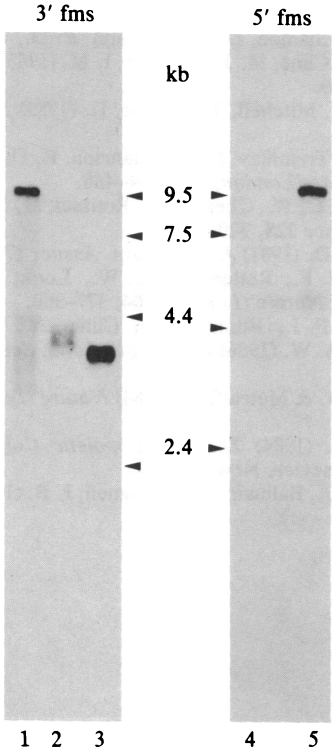

FIG. 4. RNA blot analysis. Samples (5 μg) of poly(A)$^+$ total RNA from EGV_6-T (lanes 1 and 5), human choriocarcinoma line BeWo (lane 2), normal rat aveolar macrophages (lane 3), or REF (lane 4) were separated by electrophoresis in formaldehyde/agarose gels, transferred to nitrocellulose, and hybridized to 3′ v-*fms* probe or 5′ *fms* probe as indicated.

lines were derived from tumors of epithelial origin and produced squamous cell carcinomas when injected into nude mice. Relative to normal rat cells, no gene amplification or rearrangement of cellular *fms* sequences could be detected in the tumor-derived cell lines. In addition, *fms* homologous transcripts in the transformed cells were 9.5 kb long, in contrast to normal rat c-*fms*/CSF-1 receptor mRNA, which was determined to be 3.8 kb.

The tumors from which the three cell lines that expressed the 9.5-kb *fms* homologous transcripts were derived were produced by primary tracheal epithelial cells transformed *in vitro* by MNNG. While this work reports expression of a *fms*-related gene in chemically transformed cells, this does not appear to be a general response in rat tracheal epithelial cells to carcinogen treatment; two other tumor-derived lines, one derived from MNNG-transformed cells and the other derived from γ-irradiated cells, did not express *fms*-related transcripts. In addition, lack of *fms*-related gene expression could not be correlated with the type of tumor produced by the transformed cells; of the two cell lines that did not express *fms*-related mRNA, one produced keratinizing cysts and the other produced squamous cell carcinomas.

High levels of expression of *fms* have been noted in tissues that function as sites of hematopoiesis, such as spleen and bone marrow (33), as would be expected for tissues containing mononuclear cells expressing the CSF-1 receptor. Interestingly, the McDonough strain of feline sarcoma virus (SM-FeSV) from which v-*fms* was originally isolated induces fibrosarcomas rather than hematopoietic neoplasms (34) and v-*fms* can effectively transform fibroblasts such as NIH 3T3 cells (12). Because fibroblasts have the capacity to secrete CSF-1, it has been proposed that transformation by v-*fms* may occur via an autocrine mechanism by which cells that produce the growth factor ectopically express the *fms*/CSF-1 receptor gene product. Recent experiments suggest, however, that v-*fms* is an unregulated kinase (35) and replacement of the COOH terminus of the v-*fms* gene product with the 40 COOH-terminal amino acids of c-*fms* reduces the ability of v-*fms* to transform fibroblasts (36). Therefore, autocrine stimulation may not be the primary mechanism by which the viral form of the *fms* gene transforms fibroblasts.

The possible role of a *fms*-related gene in the transformation of tracheal epithelial cells is not at present known. We speculate that the 9.5-kb mRNA expressed by the transformants may belong to a *fms*-related family of receptor molecules. RNA blots hybridized with a 3′ v-*fms* probe and rehybridized to 5′ v-*fms* probe show a much weaker 5′ v-*fms* signal relative to that observed with the 3′ v-*fms* probe (data not shown), suggesting that the 5′ end of the *fms*-related transcript is less homologous to v-*fms* than the 3′ end. This 5′ region would correspond to the amino portion of the *fms*/CSF-1 receptor, and this external binding domain would be the predicted region of least homology within a family of related receptor molecules specific for different ligands.

Interestingly, the hematopoietic cell line WEHI-3B(D$^+$), which contains receptors for granulocyte/macrophage CSF (GM-CSF) and granulocyte CSF (G-CSF) has been shown to express both a 4.1-kb *fms* mRNA and a larger *fms* homologous transcript of 8.4 kb (37). Epithelial cells have the capacity to synthesize both GM-CSF and G-CSF (38, 39), leaving open the intriguing possibility for autocrine stimulation in the transformed cells if the *fms*-related gene expressed by the EGV-T lines is the receptor for either GM-CSF or G-CSF. Future studies should therefore focus on identifying the *fms*-related gene expressed by the transformed epithelial cells and on determining whether it is related to receptors for other CSFs.

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