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Tumorigenicity of cell lines with altered lipid composition

(cultured cells/cell fusion)

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ABSTRACT **A series of closely related mouse fibroblast cell lines that differ in their content of neutral ether-linked glycerolipid and fatty acids has been used to investigate the relationship between lipid composition and tumorigenicity. Although these cell lines, derived from the same parental culture, were selected without reference to transformation or tumorigenicity, their ability to form tumors in irradiated mice was found to be closely correlated with ether-lipid content. The cell line with the highest level of ether-lipid (designated F_{40}) produces more tumors, the tumors appear more rapidly than when parental cells are injected, and the number of F_{40} cells required for tumor induction is less by a factor of ≈1000. F_{40} tumors are highly invasive, readily metastasize, and rarely regress, in contrast to the occasional benign tumors produced by the parental cell line. Cell lines that are intermediate in their lipid composition appear to be intermediate in tumorigenicity. This panel of graded cell lines provides a useful model system for both *in vitro* and *in vivo* studies on the acquisition of tumorigenicity and malignancy in cultured cells.**

A large body of literature has suggested an association between lipids and tumors (1–3). Several investigators have found elevated levels of an unusual class of compounds, neutral ether-linked lipids, in a variety of tumors in animals and man (4–9). Certain cultured cell lines of different origins that contain high ether-lipid levels have been found to be tumorigenic (10–13), but little information has been available on the tumorigenicity of closely related cell lines that differ primarily in lipid content, so that the significance of this variable could be evaluated.

In experiments designed to investigate the mechanism of cell fusion, we previously developed (from a culture of mouse L-cell fibroblasts) a series of cell lines that are highly resistant to the fusogenic effects of polyethylene glycol (PEG) (14, 15). Biochemical analysis of these cells has revealed several alterations in lipid content that appear to be directly responsible for the fusion response in these cells (ref. 16; unpublished data). Among the biochemical features associated with the PEG-resistant phenotype is a >30-fold increase in neutral ether-lipid content. In addition to the parental and the most highly lipid-altered cell lines, the selection procedure produced a graded series of homogeneous, genetically stable cell lines that span the two extremes with respect to ether-lipid content and fusibility (15, 17).

The progressively altered lipid composition of these cells provides a system for studying the association between neutral ether-linked lipids and tumorigenicity. These PEG-resistant cells were derived from a single culture of parental cells without reference to their degree of transformation *in vitro* or tumorigenicity *in vivo* (15). If ether-linked lipids are indeed a significant factor in tumorigenicity, then these cells might be expected to form tumors in proportion to their content of ether-lipid. We report here a direct correlation between ether-lipid content and the ability to form tumors in mice. Features of increased malignancy acquired by these cells in parallel with their acquisition of an altered lipid content include increased invasiveness into adjacent tissues, ability to metastasize, and decreased tumor regression.

MATERIALS AND METHODS

Cells and Media. The isolation and characterization of PEG-resistant derivatives from a parental line of bromodeoxyuridine-resistant mouse L cells (clone 1D) (18) has been described (15). Increasingly PEG-resistant cells, designated F_4, F_8, F_{16}, etc., were selected by repeated cycles of treatment with fusogenic concentrations of PEG, followed by outgrowth and serial passage of the remaining unfused cells. Cells were grown in reinforced Eagle's medium containing 4.5 g of glucose per liter supplemented with 10% fetal bovine serum (Flow Laboratories) and were periodically monitored for mycoplasma contamination by staining with Hoechst 33258 (19).

For tumorigenicity studies, cells were harvested in late logarithmic phase without protease treatment by incubation in 10 mM EDTA, washed twice by centrifugation, counted, and resuspended in phosphate-buffered saline. Cells were maintained on ice to prevent aggregation and brought to room temperature before injection; cells were >90% viable (by trypan blue exclusion).

Lipid Analysis. Analytical procedures are detailed elsewhere (ref. 16; unpublished data). Briefly, samples were extracted in organic solvents (20) and stored for short periods at −15°C under nitrogen gas. Thin-layer chromatography plates (0.25-mm silica gel 60; Merck) were prewashed in chloroform/methanol, 2:1 (vol/vol), and activated at 120°C for 1 hr before use. Samples were spotted under a gentle flow of nitrogen gas in a horizontal band and separated by a two-step procedure: (*i*) run for 11 cm in chloroform/methanol/water, 100:42:6 (vol/vol), and (*ii*) dried under nitrogen and rerun for 18.5 cm in hexane/ethyl ether/acetic acid, 80:20:2 (vol/vol). Plates were air-dried and stained with iodine vapor or sprayed with a strong oxidizing solution and charred by brief heating at 120°C. Lipid standards were obtained from Alltech (Deerfield, IL) and Sigma.

Development of Tumors. C3HeB/FeJ mice (The Jackson Laboratory) were used because the mouse L-cell line was isolated from a C3H/He mouse (21). This mouse strain was separated from the Andervont strain in 1938 and rederived by embryo transfer to a C57BL foster mother in 1948 to eliminate murine mammary tumor virus (22). No spontaneously arising tumors were observed during this study. The procedures of Sanford *et al.* (23) and Hellman *et al.* (24) were followed, with some modifications. In most experiments female mice were used, but no sex-related differences were observed when male mice were used. Mice were obtained at age 5 weeks, acclimatized to filter-top cages, and supplied with autoclaved food and water. At 7 weeks, mice were irradiated with 425 rads (whole body dose) from a ^{137}Cs source

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Abbreviation: PEG, polyethylene glycol.

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and 4–6 hr later were injected intramuscularly in the hind thigh with freshly harvested cells in 0.5 ml of phosphate-buffered saline. In some experiments, phosphate-buffered saline without added cells was injected into the opposite thigh as a control. Mice were examined weekly for the presence and size of tumors. In general, experiments were continued for at least twice as long as the time required for the latest developing tumor to arise.

Histological Preparation. Mice were killed in CO_2 gas and perfused with 10% phosphate-buffered formalin; tissue specimens were embedded in paraffin. After fixation, specimens containing bone were decalcified for 24 hr in a freshly prepared solution of 20% formic acid containing 100 g of sodium citrate per ml, followed by neutralization in two rinses of saturated LiCl (in water) and embedded in paraffin. Sections (5–6 μm) were stained with hematoxylin and eosin, dehydrated in ethanol series, and cleared in xylene.

RESULTS

Lipid Content of PEG-Resistant Cell Lines. Although all were derived from the same culture of clone 1D cells, the various cell lines differ considerably in alkyl (ether-linked) lipid content (Fig. 1). Across the series of increasingly PEG-resistant cell lines (lanes 1–5) neutral ether-linked lipids (Fig. 1 *Top*, double arrowhead) increase >30-fold, accounting for up to 5% of the whole-cell lipids in F_{40} cells (determined by scanning densitometry of charred plates). The neutral ether-linked lipid comigrated with a synthetic alkyldiacylglycerol (lane 6) and has been shown to consist of 1-*O*-alkyl-2,3-diacylglycerols of mixed chain length (16). Other differences in neutral lipid content also were observed, including increased triglyceride levels (Fig. 1 *Top*, single arrowhead), but these were less extensive than the increase in ether-lipids. Plasmalogen levels were low in these cells and unaltered between clone 1D and derivative F_{40} (not shown). Further analysis failed to reveal differences in phospholipid composition, as shown qualitatively in Fig. 1 *Bottom*. In addition to differences in ether-lipid content, these cell lines differ in fatty acid saturation (unpublished data). The ratio of saturated to polyunsaturated acyl chains increases from ≈1:1 in clone 1D cells to ≈4:1 in F_{40} cells. Intermediate cell lines are intermediate in both ether-lipid levels and fatty acid saturation.

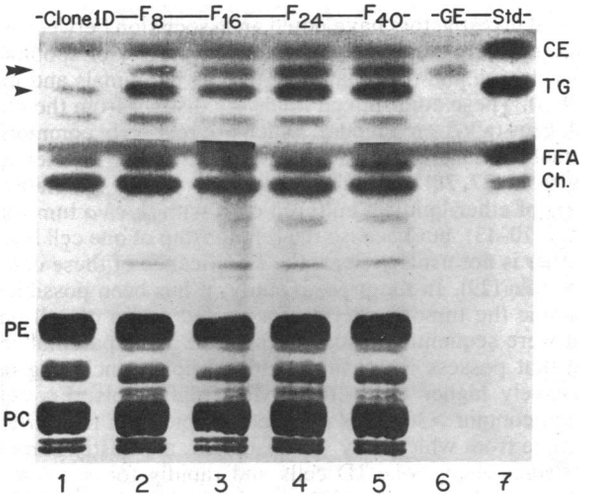

FIG. 1. Lipid-class composition of the clone 1D cell line and its PEG-resistant derivatives. Extracted lipids from 10^7 cells (lanes 1–5) were separated by a two-step procedure and exposed to iodine vapor. Lanes: 1, clone 1D; 2, F_8; 3, F_{16}; 4, F_{24}; 5, F_{40}; 6, synthetic 1-*O*-hexadecyl-2,3-dipalmitylglycerol; 7, mixture (10 μg each) of cholesterol (Ch), oleic acid (FFA), triolein (TG), cholesteryl oleate (CE), and methylolein (which comigrates with TG in this solvent system). Note the progressive increase in glyceryl ether content between clone 1D and F_{40} cells (double arrowhead). This lipid comigrates with the synthetic alkyldiacylglycerol in lane 6. Triglyceride content also increases significantly (single arrowhead). PC, phosphatidylcholine; PE, phosphatidylethanolamine.

Tumorigenicity of Lipid-Altered Cells. Because the alkyl glycerols which are prominent in F_{40} cells had been found previously in tumor tissue (4–9), mice were injected with clone 1D and F_{40} cells to assess the correlation between ether-lipid content *in vitro* and tumorigenicity *in vivo*. Fig. 2 shows the cumulative percentage of mice that developed tumors when various doses of the parental cell line (clone 1D) or the highly lipid-altered F_{40} cells were injected intramuscularly into irradiated mice. Across a wide dose range, F_{40} cells were much more tumorigenic than clone 1D cells. At high doses (10^7 cells), 100% of F_{40}-injected mice developed tumors within 1 week, whereas only 11% of mice injected with clone 1D cells developed detectable tumors. Even after 6 weeks, tumors developed in only 61% of all clone 1D-injected animals. A clear dose–response relationship was found: at each dose, F_{40} cells formed tumors more rapidly and in more animals than did clone 1D cells. In these experiments, the threshold for clone 1D-induced tumors was 10^5 cells; no tumors developed in 29 mice injected with 10^4 clone 1D cells in three experiments. By contrast, tumors were induced with as few as 100 F_{40} cells (date not shown).

No tumors were produced in two experiments in which 10^7 clone 1D or F_{40} cells were injected into 24 unirradiated mice. Although L cells were originally isolated from C3H mice, clone 1D cells have undergone extensive selection and are characterized by numerous chromosomal abnormalities (25, 26); they are no longer histocompatible with the mouse strain of origin, and irradiation is necessary.

The observed differences in tumorigenicity were highly reproducible. Table 1 shows the results of eight experiments in which a relatively high dose (10^6) of various cells were injected. Clone 1D cells induced tumors in an average 43% of mice in these experiments (SD, 11%); 84% of animals receiving 10^6 F_{40} cells developed tumors (SD, 11%). In four experiments, mice were injected with cells intermediate between clone 1D and F_{40} in ether-lipid content. Although the number of animals injected was too small to conclusively prove that the cell lines F_4, F_8, and F_{16} are progressively more tumorigenic than clone 1D, the data are consistent with this interpretation: F_8 cells caused tumor formation in 55% of injected mice (SD, 21%); and F_{16}, in 71% (SD, 20%).

These results demonstrate that (*i*) cell lines that contain higher levels of ether-lipid and saturated fatty acids produce more tumors than do cells of the parental line, and (*ii*) these tumors arise earlier and grow much more rapidly. Fig. 3 shows typical tumors induced by injection of F_{40} and clone 1D cells. Those tumors that did arise from clone 1D cells were relatively small (Fig. 3*A*), with an average maximum diameter of 1.5 cm (weight, ≈2.5 g) at 6 weeks postinjection, compared with >3.5 cm for F_{40} tumors (Fig. 3*B*; average

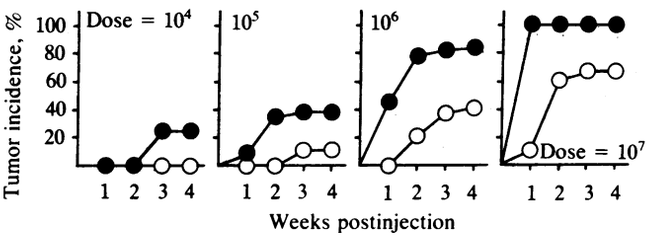

FIG. 2. Tumor development in mice injected with various doses of clone 1D or F_{40} cells. C3HeB/FeJ mice were irradiated and injected intramuscularly and examined weekly for tumors. The cumulative percentage of mice that had developed tumors by the week indicated is presented for clone 1D (○) or F_{40} (●) at doses between 10^4 and 10^7 cells.

Table 1. Tumor induction by clone 1D cells and PEG-resistant derivatives*

Cell line	Ratio of tumor-bearing mice to number infected in experiment no.							
	1	2	4	5	6	8	9	11
Clone 1D	3/6	5/9	2/5	5/9	3/6	3/9	2/9	7/15
F_4					3/10			
F_8					3/8			8/12
F_{16}		8/9	4/5		3/6			
F_{40}		9/9	4/5	7/9	4/6	8/9	8/9	7/9

*Total number of mice developing tumors of any size after intramuscular injection of 10^6 cells into irradiated C3HeB/FeJ mice.

weight, 12 g). The latter tumors averaged ≈30% of the whole body weight and, in extreme examples, reached >60% of body weight. Considering only those tumors that reached at least 2-cm diameter, we found that the mean time to attain this size was 6.6 weeks (SD, 2.3 weeks) for clone 1D tumors and 3.4 (SD, 1.1) for the F_{40} tumors. These marked differences between clone 1D and F_{40}-induced tumors were retained even when both tumors were present in the same mouse, i.e., in mice injected with clone 1D cells in one hind leg and F_{40} cells in the other (not shown).

The less frequent, smaller tumors produced by clone 1D cells also regressed more often than F_{40} tumors (Table 2). In five experiments, regression was observed in 75% of the clone 1D tumors, but in only 31% of F_{40} tumors. The time required for regression varied between 1 and 5 weeks after reaching maximum size. In general, those tumors that regressed rapidly had also arisen rapidly.

Tumor Pathology. Primary tumors were found at the site of injection, forming a single solid mass, often encapsulated, with extensive invagination. Histological preparations of such tumors were highly homogeneous, with a high mitotic index (Fig. 4*A*). Internal necrosis often was found in larger tumors. L cell-induced tumors are usually identified as fibrosarcomas (23, 24, 26), although there is a lack of fibrous material. More than any classical tumor, however, these tumors resemble L cells grown in culture, with prominent nucleoli and somewhat more rounded morphology than is typical of fibroblastic cells.

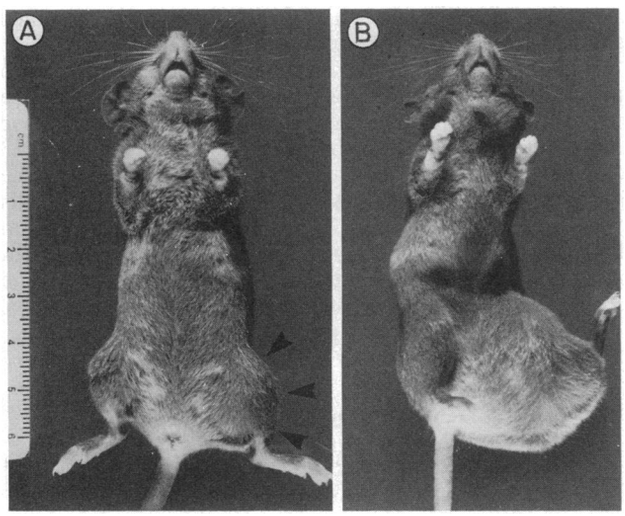

FIG. 3. Gross pathology of tumors induced by clone 1D or F_{40} cells. Mice were injected with 10^6 cells and sacrificed 6 weeks later. (*A*) Clone 1D cells injected into the left hind leg. (*B*) Similar injection of F_{40} cells. Fewer than 50% of clone 1D-injected mice develop tumors from 10^6 cells (see Fig. 2), and those tumors that develop are small (arrowheads) compared with the large, highly malignant F_{40} tumors.

Table 2. Tumor regression*

Cell line	Total mice	Total no. tumors (% of total mice)	Total no. regressed (% of total no. tumors)
Clone 1D	39	16 (41%)	12 (75%)
F_{40}	33	29 (91%)	11 (38%)

*All mice were irradiated with 425 rads and injected intramuscularly with 10^6 cells. Experiments in which some animals were sacrificed before tumors had a chance to regress are not included in this analysis.

Both clone 1D and F_{40}-induced tumors readily invaded muscle tissue; Fig. 4*B* shows tumor cells separating muscle fibers. Examination of the tissues surrounding tumors provided further evidence of the greater malignancy of F_{40}-induced tumors, as in the longitudinal sections through the legs of mice bearing tumors induced by 10^6 clone 1D or F_{40} cells (Fig. 4 *C–F*). The clone 1D tumors (Fig. 4 *C* and *E*) are rare examples of large tumors produced by this cell line. The tumor in Fig. 4*C* is adjacent to the head of the femur and wraps the bone shaft, but the bone, cartilage, and marrow have not been invaded. Typical F_{40} tumors are seen in Fig. 4 *D* and *F*. In contrast to the clone 1D tumors, extensive invasion of bone marrow is evident, eroding the shaft and internal bony spicules. Collapse of the long bones due to extensive tumor invasion is common in mice injected with either F_{16} or F_{40} cells but has not been seen with clone 1D tumors.

Examination of mice bearing clone 1D-induced tumors failed to yield any consistant abnormalities other than the primary leg tumors, and no metastases were found in any clone 1D-injected mouse. In striking contrast, F_{40} tumors frequently metastasized to the lung. Fig. 5*A* shows typical metastases in a mouse injected with 10^6 cells 10 weeks prior to sacrifice. The morphology of these metastases is very similar to that of the primary leg tumor (cf. Fig. 4*A*) and distinct from lung metastases induced by murine mammary tumor virus in C3H mice not rederived from a murine mammary tumor virus-negative foster mother (Fig. 5*B*).

DISCUSSION

Several investigators have noted an association between elevated neutral ether-lipid content and a variety of spontaneously arising and transplantable tumors in animals and man (2, 4–9). These ether-linked lipids are distinct from the plasmalogens (alk-1-enyl lipids), which are relatively common in normal mammalian tissue and also occasionally are elevated in tumors (27, 28). There have been attempts to correlate the levels of ether-lipids in cultured cells with *in vivo* tumorigenicity (10–13), but because the relationship of one cell line to another is not usually clear, the significance of these data is uncertain (29). In the present study, it has been possible to examine the tumorigenicity of a graded series of cell lines that were sequentially isolated from the same parental cells and that possess increasingly altered lipids, including progressively higher levels of alkyldiacylglycerols. F_{40} cells, which contain >30 times more ether-lipid than the parental cell line from which they were isolated, are vastly more tumorigenic than clone 1D cells and rapidly form tumors in more animals and at much lower doses than does clone 1D. Karyotyping of tumors induced by injections of either cell line (not shown) indicates that these tumors develop from the injected cells. F_{40} tumors usually grow to large size, rarely regress, and are highly malignant, in contrast to the smaller, relatively benign tumors that occasionally arise after injection of clone 1D cells. Furthermore, F_{40}-induced tumors readily invade adjacent muscle and bone and frequently develop lung metastases. The presence of metastases in these F_{40}-injected animals is noteworthy; to our knowledge this is the first reported instance of metastasis of an L-cell derivative.

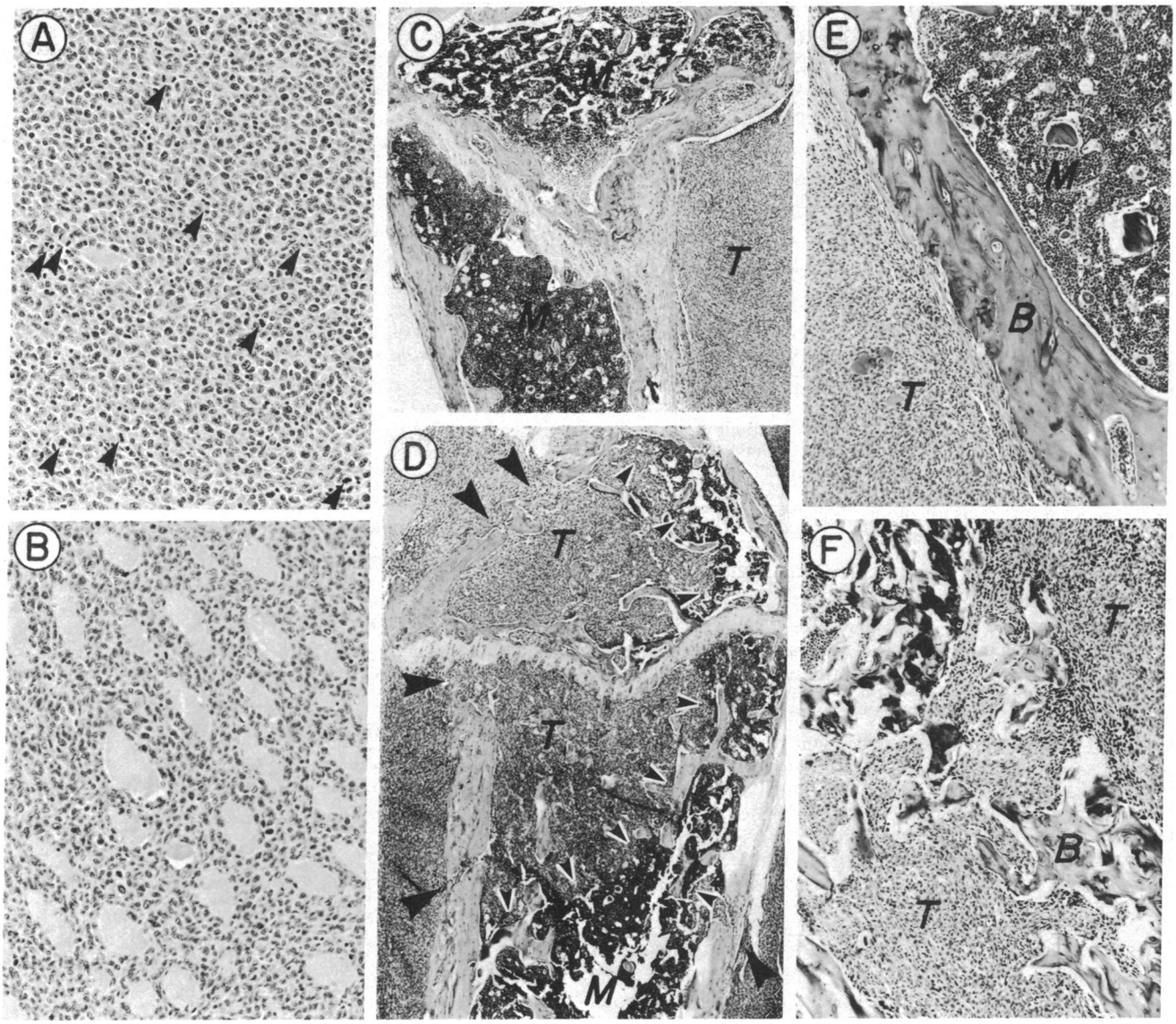

FIG. 4. Tumor histology. (*A*) Section through a typical tumor induced by injection of F_{16} cells, illustrating homogeneous tissue with a high mitotic index (arrowheads). (*B*) Invasion of muscle tissue by an F_{16}-induced tumor; the lighter-staining ovoid structures are cross sections through individual muscle fibers. (*C*) Longitudinal section through the head of a femur from a mouse injected with 10^7 clone 1D cells. (*D*) Longitudinal section through the tibia of a mouse injected with 10^6 F_{40} cells. Both the clone 1D and F_{40} tumors completely wrap around the bone shaft. Only the F_{40} tumor is invasive, however, eroding the bone at many points (large arrowheads) and filling much of the marrow cavity (small arrowheads). (*E*) Higher magnification of a clone 1D-induced tumor and the adjacent femur, showing normal tumor, bone, and marrow. (*F*) Section through the femur of a typical F_{40}-tumor bearing mouse, demonstrating the highly invasive capacity of F_{40} tumors. *M*, marrow; *B*, bone; *T*, tumor.

Sufficient numbers of mice have not yet been examined to unequivocally determine the tumorigenicity of cells that are intermediate between clone 1D and F_{40} in their ether-lipid content, e.g., F_4, F_8, and F_{16} cells, but the available data suggest that these cells are intermediate in their ability to induce tumors. According to most measures of malignancy (growth rate, invasive capacity), F_{16} is very similar to F_{40}. With respect to metastasis, however, F_{40}-injected mice appear to develop metastases more frequently.

Two biochemical alterations are particularly striking in the cell lines under study: increased ether-lipid content and increased saturation of fatty acid chains. There is no definitive evidence to indicate which of these factors is of primary importance for tumorigenicity. However, the acyl-chain composition of tumors appears to reflect serum fatty acid content (data not shown), suggesting that the ether-linked lipid may be more directly associated with tumor formation. Unlike most neutral lipids, ether-linked compounds appear to be preferentially localized in cell membranes (16, 30–32). Thus, they may mediate membrane-associated events, either directly or through association with specific cellular enzymes or structural proteins.

F_{40} cells were originally selected for resistance to cell fusion induced by PEG, and these mutants are distinguished from clone 1D cells by a variety of membrane-associated properties. The PEG-resistant mutants are particularly sensitive to infection and cell fusion induced by enveloped viruses (unpublished data). Logarithmic phase growth of all of these cell lines is similar, but the overall growth rate of F_{40} cells in culture is more rapid than clone 1D due to a decreased lag before reaching logarithmic phase and decreased growth inhibition at confluence (16).

These studies have demonstrated an association between specific lipid alterations of cells grown in culture and their

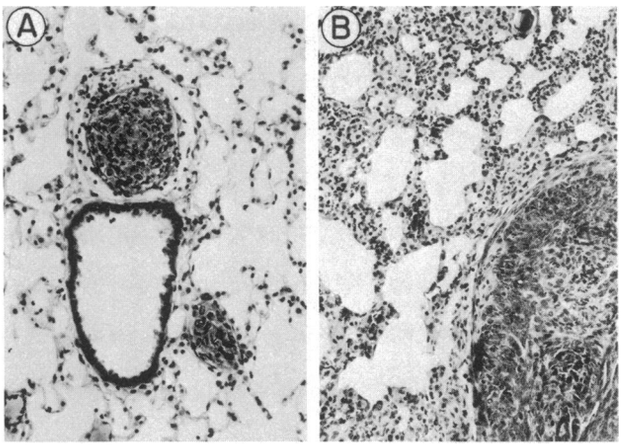

FIG. 5. Metastasis of F_{40}-induced tumors. (*A*) Lung section of a mouse bearing an F_{40} tumor, with metastases of similar morphology to the primary tumor (cf. Fig. 4*A*). (*B*) Pulmonary metastasis from a primary mammary adenocarcinoma. The metastatic lesion in the F_{40} tumor-bearing mouse is clearly distinct from the lobular tissue characteristic of mammary adenocarcinoma.

tumorigenicity *in vivo*. Although the mechanisms involved in a relationship between lipid composition and tumorigenicity remain obscure, the present system offers many opportunities for probing such interactions. Several biochemical characteristics of these cells have been determined, and these observations can now be compared with the biochemical properties of tumors induced by the cell lines under study. Preliminary analysis of those infrequent tumors that are induced by clone 1D cells reveals an ether-lipid content similar to that of F_{40} cells rather than the original cells that were injected. Recently, we have succeeded in controlling the ability of these cells to fuse by the manipulation of their lipid content *in vitro* (unpublished data). The ability to predictably alter the lipid content of these cells in culture may be useful in probing interactions that occur in the mouse. Such studies also suggest a variety of parallel experiments that may be carried out *in vivo*. Analysis of oncogene expression in this system and studies on lipid content of other cells before and after transformation may clarify the relationship between lipid composition and tumorigenicity.

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