Organ Colonization Pattern of Retinoic Acid-treated and -untreated Mouse Embryonal Carcinoma F9 Cells

Benedetto Terrana, Dario Rusciano, and Lorenzo Pacenti
Scavo Research Centre, Via Fiorentina 1, 53100 Siena, Italy

ABSTRACT

The mouse embryonal carcinoma cell line F9 differentiates into parietal endoderm cells after a 3-day exposure to retinoic acid and dibutyryl cyclic AMP. Using the experimental metastases assay, we investigated the organ colonization properties of RA-treated and -untreated populations of F9 cells. The results show that untreated F9 cells colonize the liver with a high degree of specificity while the treated populations colonize the lungs. Cells derived from a lung colony colonized only the liver unless they were treated with RA. However, removal of the inducer from culture of differentiated cells did not cause reversal of the lung colonization potential. Our observations also indicate that it is unlikely that lung colonization is due to cell aggregation or to interaction between differentiated and undifferentiated cells. Taken together, these results suggest that RA induces the observed change of organ colonization properties of F9 cells.

INTRODUCTION

Mouse teratocarcinomas are highly malignant tumors (1) which, since the development of transplantable tumor lines (2,3), have been used for the study of problems related to both tumorogenesis and differentiation during early embryogenesis (1,4). A number of both multipotent and nullipotent EC3 cell lines, i.e., the stem cells of these tumors, have been developed. In vivo the nullipotent lines give rise to undifferentiated tumors while multipotent lines give rise to solid tumors containing derivatives of all three embryonal tissues (1). In vitro multipotent EC cells can be induced to differentiate while nullipotent EC cells are either incapable of differentiation or they can give rise to only a limited type of progeny (5). In all cases studied, EC cells have been found to possess a high degree of organ specificity (6-9). Thus, these cells might provide a good model system to study the homing mechanisms relevant both to the metastatic spread of tumors and lymphocyte homing (7). Furthermore, the fact that differentiation results in antigenic and enzymatic changes while the cells might remain tumorigenic should enable one to study the relationship between such changes and organ colonization properties. In order to investigate these possibilities, we studied the organ colonization properties of F9 cells in relation to differentiation.

F9 EC cells are nullipotent in vivo (10) while in vitro they can be induced to differentiate by treatment with RA and DBC (11,12). RA- and DBC-treated F9 cells assume the morphology of parietal endoderm cells and express markers typical of these cells, such as plasmagmin activator, collagen type IV (12), the intermediate filament proteins keratin, endo A and B (13), and laminin (14). The surface glycoconjugates of F9 cells also undergo considerable changes with differentiation as evidenced by the loss of expression of the stage-specific embryonal antigen SSEA-1 (15) and the extensive reduction of embryoglycan (16). The results presented below show that F9 EC cells are highly specific for liver colonization while differentiated cells obtained by treatment with RA colonized the lungs of syngeneic animals.

Our data also show that the change in organ colonization requires the presence of RA, and is not reversible 3-days after removal of the inducer. We found no evidence that lung colonization property is due to interaction between differentiated and undifferentiated cells.

MATERIALS AND METHODS

Cell Culture and Differentiation. F9 cells used in these studies were obtained from Dr. P. H. Atkinson. Stock cultures were maintained in uncoated plastic bottles (Falcon) by seeding at 10^6 cells/cm^2 in Dulbecco's modified minimum essential medium supplemented with 10% FBS. Stocks were passed by trypsinization every 2 to 3 days. F9ACC19 and PYS-2 cells were obtained from Dr. H. Axelrod (Wistar Institute) and maintained in Dulbecco's modified minimum essential medium supplemented with 10% FBS. Liver and lung nodules to be explanted were removed from the animals and sterilely transferred to culture medium. When possible, the capsule was removed and the tumor tissue broken up with tweezers. This suspension was then gently pipetted with a 5 or 10 ml pipet to disperse clumps, dispensed in 60-mm culture dishes, and incubated overnight. The next day the medium was changed to remove all unattached cells and tissue clumps. The cells were allowed to grow and frozen without further transfer. Additionally some cells were transferred once and then frozen. Cell cultures to be differentiated were seeded at 10^5 cells/cm^2 and 10-12 h later the medium was replaced with medium containing 0.1 μM RA (Sigma) and 1 mM DBC (Sigma) as described (11). In those cases where reversal was attempted, the medium was changed to fresh inducer-free medium at the end of the third day after the start of induction.

Organ Colonization Assay. In these studies we used the organ colonization assay essentially as described by Fidler (17). F9 cells were seeded as for stock cultures and after a 1- to 2-day growth period, the flasks were flooded with 0.25% trypsin for a few seconds and then incubated at 37°C. After total exposure for 3 min to trypsin, medium containing 20% FBS was added to the flask and pipetted several times to break up clumps. The cell suspension was centrifuged for 5 min at 1600 x g and the pellet resuspended in Hank's balanced salt solution. The cells were then counted with a hemocytometer and diluted as needed. Viability was determined by the Trypan blue exclusion method and generally found to be between 85 and 90%. Eighty to 90% of the cells were single, double, or triple cells aggregates with the latter being a small proportion of the total. If clumps were present, they were removed by filtration through gauze. Cell suspensions of differentiated cells obtained by a 3-day exposure to RA were prepared as described for F9 cells, except that they were always filtered through several layers of gauze to remove clumps. Viability and the distribution of single, double, and triple cell aggregates were also similar to that found for F9 cells. The cell suspension to be injected was maintained in agitation by gentle magnetic stirring at room temperature and 0.25-0.3 ml containing the desired number of cells was injected with an insulin syringe equipped with a 25-gauge needle in the lateral tail vein of syngeneic animals (age, 9-14 weeks). Viability, cell number, and presence of aggregates were checked at the end of the injection procedure and found to be unchanged. Before injection the animal's tail was gently warmed by dipping it in water at 41°C for about 30 s. This was found to be sufficient to swell the vein and render it more easily identifiable. The treated animals were killed 3 weeks after injection and autopsied.

In the reversal experiments, cells were induced to differentiate as described above. At the end of the third or fourth day, the medium was replaced with inducer-free medium and the incubation continued. After 1, 2, and 3 days in these conditions, the cells were removed and animals...
were given injections as described above. To increase tumor incidence the mice were given inoculations of \(10^6\) cells.

In the mixing experiments, cells obtained from parallel treated and untreated cultures were each used to give injections to a group of control animals at \(5 \times 10^5\) cells/mouse. These two populations were then mixed and a group of mice were given injections so that each animal received \(5 \times 10^5\) treated and \(5 \times 10^5\) untreated cells. The animals were analyzed as described above.

All the animals used in these experiments were strain 129/Sv-ter and were maintained in our animal facilities by brother to sister mating starting from animals obtained from Dr. L. C. Stevens (The Jackson Laboratory, Bar Harbor, ME).

Preparation of Cell Surface Glycopeptides. Undifferentiated cells were seeded at \(2 \times 10^4/cm^2\) and 24 h later labeled with 4 \(\mu\)Ci/ml of \(^1^4\)C-labeled fucose (New England Nuclear, specific activity, 59.7 mCi/mmol). Differentiated cells obtained as described above were labeled with 10 \(\mu\)Ci/ml of \(^3^H\)-labeled fucose (New England Nuclear, specific activity, 84 Ci/mmol) starting at 60 h after induction. 24 h later, the cells were detached in \(Ca^{2+}\)- and \(Mg^{2+}\)-free phosphate-buffered saline (NaCl, 0.137 mM; KCl, 2.6 mM; KH\(_2\)PO\(_4\), 1.5 mM; Na\(_2\)HPO\(_4\), 2H\(_2\)O, 6.5 mM) containing 27 mM EDTA, washed once by centrifugation in \(Ca^{2+}\)- and \(Mg^{2+}\)-free phosphate-buffered saline, and then extensively digested with 40 mg of pronase (Boehringer Mannheim, from Streptomyces griseus) in 4 ml of digestion buffer (0.05 M Tris-HCl, pH 8.0, containing 0.01 M CaCl\(_2\)) (18) at 37°C for 24 h under a toluene layer. Fresh Pronase (40 mg) in 2 ml of digestion buffer were added 24 and 48 h later. Digestion was terminated after 72 h by heating at 100°C for 10 min. Digested material was clarified by centrifugation and the supernatant was lyophilized. This was then applied to a column (1.5 x 70 cm) of Sephadex G50 (extra fine) and eluted with 0.05 M ammonium acetate buffer, pH 6.0. Fractions (1.5 ml) were collected, and 10-ml aliquots counted for radioactivity with Biofluor (New England Nuclear) in a \(\beta\) scintillation counter (Beckman).

The glycopeptides eluted in the void volume (fractions 34-40, undifferentiated cells; fractions 32-39, differentiated cells) were collected, lyophilized, and an aliquot was applied to a column (1.5 x 95 cm) of Sephadex G100 (extra fine), which had been equilibrated in ammonium acetate buffer. Fractions (1.5 ml) were collected and counted for radioactivity.

It has been shown (19) that fucose-labeled glycopeptides obtained as described above are identical to glycopeptides derived from isolated membranes of F9 cells.

Immunofluorescence. Control cells and cells to be treated with RA were seeded as described above on 18 x 18-mm coverslips. Control cells were used for immunofluorescence after 1-2 days while differentiated cells were used after a 3-4-day exposure period to RA. The cells were fixed in acetone at \(-20^\circ\)C, rinsed and incubated with anti-SSEA-1 antibody at room temperature for 30 min, rinsed again and incubated with the secondary antibody at room temperature for 30 min. Anti SSEA-1 was a kind gift of Dr. B. Knowles (Wistar Institute of Anatomy, Philadelphia, PA) and was used at a dilution of 1:100 and 1:200. Fluorescein isothiocyanate goat anti-mouse IgM (Cappel Worthington) was used at a dilution of 1:40. This dilution gave no background fluorescence on cells that had not been treated with primary antibody.

RESULTS

Organ Colonization Pattern of F9 Cells. Exponentially growing F9 cells were removed by trypsinization and injected in the lateral tail vein of syngeneic animals as described (“Materials and Methods”). Preliminary experiments indicated that in most of the animals that developed tumors 21 days after the injection, these were located in the liver (Fig. 1A). However, more males developed tumors compared to females (not shown). Similarly, s.c. implanted F9 cells (\(10^6/animal\)) were more tumorigenic in males (not shown). Such differential tumorigenicity might reflect the origin of F9 cells which were derived from teratocarcinoma 0T76050-B (10) developed from an embryo transplanted to the testis of an adult male (2).

Fig. 1. Liver and lung tumors obtained from untreated and RA- and DBC-treated cells. A, liver tumors obtained by injection of F9 cells; B, a lung nodule obtained after injection of differentiated F9 cells.

Based on these observations, further studies of the organ distribution of tumors were conducted in males. The results presented in Table 1 show that tumorigenicity was dependent on the inoculum size, since it was low at around 25-30% when less than \(10^5\) cells were injected and increased to about 70% when \(1.5-2 \times 10^5\) cells inocula were used. Two different groups of animals (9-20 animals/group) of comparable age were given injections each inoculum size. A comparison of the results obtained with each group of animals showed that tumorigenicity varied from 30 to 90% at the highest inocula while it was between 20 and 60% when less than \(10^5\) cells were injected per animal (not shown).

In spite of these differences in tumorigenicity, the organ distribution of tumors seemed to be highly selective since in all experiments 70-100% of the animals bearing tumors presented liver nodules (Table 1). The overall result was that in 85% (44 of 52) of tumor bearing mice included in Table 1, the tumors were located in the liver. In contrast, the kidneys were involved in 15% (8 of 52) and any other organ in less than 4% of the tumor bearing animals. These results suggest that F9 cell tumor formation is highly selective towards the liver. The liver nodules ranged in size from a few millimeters to 1 cm or more and the larger nodules were often vascularized. The number of tumors found in the liver ranged from one to 13 and in the cases with several colonies per liver the colonies were of different sizes (Fig. 1A). As is evident from Table 1, the number of colonies formed per liver appeared to increase with the inoculum size. In the few cases in which tumors were found in two organs (see Table 1, footnotes), the liver was one of the organs always involved, and the extraneoplastic nodules were generally small (4 mm in diameter). Although it is conceivable that the small
F9 CELLS EXPERIMENTAL METASTASIS

Table 1  Tumorigenicity and organ distribution of tumors obtained by tail vein injection of F9 cells in syngeneic mice

<table>
<thead>
<tr>
<th>Cells/mouse</th>
<th>Tumorigenicity</th>
<th>Liver</th>
<th>Peritoneum</th>
<th>Testis</th>
<th>Lung</th>
<th>Kidney</th>
<th>s.c.</th>
<th>Spleen</th>
<th>Pancreas</th>
<th>Colonies/ liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 x 10⁴</td>
<td>7/26 (26%)</td>
<td>7/26</td>
<td>1/26</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>7.5 x 10⁴</td>
<td>7/23 (30%)</td>
<td>5/23</td>
<td>2/23</td>
<td>1/23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.2</td>
</tr>
<tr>
<td>1.5 x 10⁵</td>
<td>15/22 (68%)</td>
<td>15/22</td>
<td>3/22</td>
<td>6/34</td>
<td>2/34</td>
<td>2/34</td>
<td>1/34</td>
<td></td>
<td></td>
<td>4.4</td>
</tr>
<tr>
<td>2.0 x 10⁴</td>
<td>23/34 (67%)</td>
<td>17/34</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Tumorigenicity and organ distribution are expressed as the fraction of inoculated mice bearing tumors at any site and in each indicated organ, respectively.

* One of these two mice also had liver nodules.

* All three mice had liver nodules.

* Three mice had nodules in liver and kidney, one in liver and s.c., one in spleen and s.c., and one in lung and kidney. All other animals had a single organ affected.

Fig. 2. Morphology of undifferentiated and differentiated F9 cells compared to cells derived from liver and lung nodules. A, F9 EC cells; B, F9 cells 3 days after treatment with RA and DBC; C, cells derived from liver nodules; D, cells derived from lung nodules (DFLu). (Magnification 150 x).

extrahepatic colonies and the small nodules in livers with several growths might derive from the metastatic spread of the larger liver nodules, the possibility cannot be excluded that these small colonies arose as slowly growing tumors from cells present in the population initially injected. Since EC cells from different laboratories may behave differently, we tested the organ colonization properties of F9 cells obtained from Dr. A. J. Levine (Princeton University). The results obtained (not shown) were identical to those described above.

We have isolated and cultured cells from some of the liver nodules. As shown in Fig. 2C the cells have the same morphology as the original F9 cells (Fig. 2A). The growth pattern of these cells was also indistinguishable from that of F9 cells.³

Organ Colonization Properties of RA-treated F9 Cells. To induce differentiation, F9 cells were treated with 0.1 μM RA and 1 mM DBC as described ("Materials and Methods"). As markers of differentiation we followed the expression of the stage-specific embryonal antigen-1 (SSEA-1) and surface glycoproteins (see below). The first morphological evidence of differentiation is apparent during the second day of treatment when the cells begin to flatten out and move apart. Roughly at about 72 h after the beginning of the treatment, most of the cells in the culture have assumed the morphology typical of endoderm cells (Fig. 2B) (11, 12). Treated and untreated cultures were tested for the expression of SSEA-1 antigen by indirect immunofluorescence ("Materials and Methods"). F9 EC cells express considerable amounts of this antigen on their surface (Fig. 3A) but, as shown in Fig. 3B, after 3 days of growth in RA the expression of this antigen is drastically reduced (15).

Differentiated F9 cells thus obtained were injected in syngeneic mice at a dose of 1.5 x 10⁵/mouse and the animals analyzed 3 weeks later as described ("Materials and Methods"). The results presented in Table 2 show that 50% (21 of 43) of the animals inoculated with RA-treated cells developed tumors. Thus, tumorigenicity of these cells is lower than that of the parent F9 EC cells since inoculation of 1.5 x 10⁵ differentiated cells results in fewer animals bearing tumors (Tables 1 and 2) and fewer tumor colonies per organ involved compared to F9 cells. That these differentiated cells remain tumorigenic is not surprising since other teratocarcinoma-derived cells expressing the markers typical of parietal endoderm cells such as PYS-2 (20) and F9ACC19 (21) are tumorigenic.

Table 2 also shows that, although 21 of 43 injected animals developed tumors, only 2 animals presented growths in the liver while 19 mice presented exclusively lung nodules. The lung colonies (Fig. 1B) were generally small, ranging from 1 to 4 mm in diameter, and few in number, ranging from one to three per lung. The same shift in organ distribution of tumors was observed in females (not shown). One of the lung nodules was explanted and grown in vitro. The cells obtained (DFLu), though negative for the expression of SSEA-1 (Fig. 3, C and D), appeared to be undifferentiated since they showed the typical morphology of F9 cells (Fig. 2D), and they present clearly distinguishable nucleoli typical of EC cells (22). Furthermore, histological evidence suggests that lung nodules are

³ B. Terrana, and L. Pacenti, unpublished data.
entirely made up of EC cells.* In keeping with the above, differentiation could be induced in these cells by treatment with RA and DBC (see below). Thus, it is possible that lung nodules resulted from the selection of a subpopulation of SSEA-1-negative F9 cells that might not respond to RA, rather than being a result of induction with RA and DBC. To test this possibility, two groups of animals were injected with untreated DFLu cells and with DFLu cells after a 3-day exposure to RA and DBC (“Materials and Methods”). DFLu cells treated with RA show the same morphological changes occurring in F9 cells (not shown). The injected animals were analyzed as described above. The results (Table 2) show that untreated lung nodule-derived cells did not retain the property to colonize the lungs. Similar to the parental F9 cells, lung colonization was induced by treatment with RA and DBC. These results show that the property of lung colonization depends on treatment with RA and DBC and it is not due to the selection of a preexisting population of SSEA-1-negative cells that do not respond to RA. Preliminary evidence suggests that exogenous cyclic AMP is not necessary to induce the change in organ colonization pattern, since cells treated with only RA also colonized the lungs preferentially (not shown).

Results similar to those described above (Table 2) were obtained with cells that had been obtained from Dr. A. J. Levine.

Origin of Lung Colonies. Among the questions posed by the observations described above are those concerning the mechanism of lung colonization and the nature of the cells that give rise to the lung nodules. In fact, there are several ways in which one could explain the colonization of the lungs by the treated population. It is possible that lung colonization is due to mechanical trapping since this organ is the first capillary bed encountered by the cells injected in the tail vein. However, we think this is unlikely for the following reasons. We always injected populations that were made of essentially single cells. The diameter of the differentiated cells after trypsinization does not appear to be considerably larger than that of the untreated population. No aggregation occurred during the injection procedure with either the treated or the untreated cells. However, it is possible that these cells might aggregate after entering the circulation. To test this possibility, we measured the capacity of differentiated cells trypsinized in the same way as for the cells that were injected (“Materials and Methods”) to aggregate in mouse serum. We found no difference in the number of cells present in the form of aggregates between RA-treated and control cells when they were suspended in mouse serum at either $10^5$ or $10^6$/ml (not shown). The results were unchanged when both cell types were suspended together, each at $10^4$ (not shown). These results agree with those of Takeichi et al. (23) who have shown that F9 cells do not aggregate when they are trypsinized in the absence of calcium.

Another possibility is that lung nodules could arise from partially and/or reversibly differentiated cells, which revert to the undifferentiated phenotype after arresting in the lungs. It has been reported that dedifferentiation is possible in vitro in the EC cell line 311 exposed to RA for 2 days (24). Consequently, we tested the possibility that the lung colonization property acquired during a 3-day exposure to RA is reversible after removal of the inducer. F9 cells were induced to differentiate as described above; after 3 days the cultures were shifted to inducer-free medium and mice injected 1, 2, and 3 days later. The morphology of the cells under these conditions is shown in Fig. 4.

As mentioned above, not all of the cells appear to have differentiated after 3 days in RA and some cells with EC cell
The fields shown are intended to illustrate the different types of colonies and cells seen in this culture and do not reflect their quantitative proportion. Arrows, colonies with EC-like morphology. (Magnification 100 x).

The great majority of cells still maintained a typical endoderm morphology, while small colonies with EC cell morphology became evident (Fig. 4B). With time, colonies with EC-like cells increased in size (Fig. 4D), although the majority of the population was still made up of cells with endoderm morphology. The organ colonization properties of cells derived from differentiated cultures that had been grown in inducer-free medium for 1, 2, or 3 days is shown in Table 3. The results of the autopsies carried out 3 weeks after the injection show that these cells still colonized the lungs, even after 3 days of culture in inducer-free medium. Tumorigenicity and the number of colonies developed per lung were very similar to those obtained with control cells after 3 and 4 days of treatment (Table 3). However, it should be pointed out that while the proportion of mice developing tumors remained high at the high cell dosages used in these experiments, the number of colonies obtained per lung was variable. These results suggest that reversal of the lung colonization properties acquired after exposure to RA either does not occur in vitro, or it takes more than 3 days. Furthermore, it should be pointed out that the results in Table 3 imply that lung colonization is not likely to be due to a direct effect of RA on cell surface. The observation that cells with EC morphology increase in the reversal cultures (Fig. 4) contrasts with the observation that this population colonizes only the lungs. A possible explanation for this apparent contradiction might be that lung colonies are derived from the EC-like cells by way of some interaction with the differentiated cells (25). To test this hypothesis, animals were injected with a 1:1 mixture of cells from a treated population and from an untreated population (see "Materials and Methods"). The results of such an experiment, presented in Table 4, clearly indicate that the mixed population colonized both liver and lungs, while the control populations gave the expected results (compare Table 4 with Tables 1 and 2). Lung and liver colonization patterns of the mixed populations were compared to those of treated (lung) and untreated (liver) populations respectively using the Mann-Whitney U test. No significant differences ($P = 0.002$) were seen between either lung ($U = 59$) or liver ($U = 45$) colonization of the mixed population compared to the respective control groups. The tables given by Siegel (26) were used to determine the expected values of $U$ for $n_1 = 7$ and $n_2 = 20$. Thus, these results do not support the hypothesis that the bulk of lung colonies are formed from undifferentiated cells via interaction with differentiated cells.

### Table 3. Reversibility of lung colonization potential of RA/DBC-treated F9 cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Time in RA/DBC (days)</th>
<th>Time after removal of inducer (days)</th>
<th>Organ distribution</th>
<th>Colonies/lung</th>
</tr>
</thead>
<tbody>
<tr>
<td>F9</td>
<td>3</td>
<td>23/25</td>
<td>Liver: 0/23</td>
<td>10.0</td>
</tr>
<tr>
<td>F9</td>
<td>4</td>
<td>8/8</td>
<td>Lung: 0/23</td>
<td></td>
</tr>
<tr>
<td>F9</td>
<td>3</td>
<td>1</td>
<td>Other: 0/23</td>
<td></td>
</tr>
<tr>
<td>F9</td>
<td>3</td>
<td>10/10</td>
<td>Colonies:</td>
<td></td>
</tr>
<tr>
<td>F9</td>
<td>3</td>
<td>0/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F9</td>
<td>3</td>
<td>10/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F9</td>
<td>3</td>
<td>0/10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Organ distribution and tumorigenicity are defined as described in Table 1.
* These are the cumulative results of three different experiments.
* In this case alone, the animals were injected with 7.5 x 10^6 each.

Changes in Surface Glycosylation during Differentiation. To study the surface glycopeptides of F9 EC cells and their differentiation product, the cells were labeled with 3H- or 14C-fucose and surface glycopeptides prepared as described ("Materials and Methods"). The elution profile of the Sephadex G50 column (Fig. 5A) clearly shows that, in agreement with previous observations (16), cells labeled between the third and fourth day of treatment with RA express very little of the high molecular weight glycopeptides which are abundant on the surface of F9 EC cells (16, 18). Fractions eluted in the void volume (see "Materials and Methods") were collected and run on a column of Sephadex G-100. The elution profile of this column (Fig. 5B) shows that the glycopeptides from RA-treated cells elute as two peaks, one excluded and one included, while the material from F9 EC cells contains only small amounts of the excluded peak. Thus our data indicate that the high molecular weight glycopeptide typical of F9 cells consists of at least two main components and that although the total amount of these glyco-
colonization by teratocarcinoma cells, namely that F9 EC cells
induced F9 cells in syngeneic mice (9) were found to colonize the ovary. The latter cells also
home preferentially to the liver (6). The data we present here describe yet another case of organ specificity of
colonization by F9 cells. We have also shown that, in spite of the high degree of
differentiation, a shift can also be observed in the relative proportions of the two components. Recently, we found a similar glycopeptide (27) on the surface
of F9 cells (28). Similar conclusions have been reached in a murine system (29, 30). Furthermore, the fact that our cells acquire the capacity to colonize the lungs only after treatment with RA suggests that only tumor cells endowed with specific properties may succeed in the colonization of the first capillary bed encountered.

The cell surface is thought to play an important role in defining the organ specificity phenomena probably involved in the homing of metastatic cells (31, 32) and lymphocytes (33). The cell surface of F9 cells has been extensively studied and it is characterized by the presence of embryonal antigens (34, 35), lectin receptors (36, 37), specific glycoproteins (23), and glycolipids (38). Among the best studied structures of F9 cell surface are the embryoglycans (39) and SSEA antigens (40, 41) also typical of other EC cell lines. Both of these surface components are carbohydrate in nature, containing terminally linked galactose residues (39-41).

Recently a galactose binding protein has been described in mouse liver that could be involved in liver colonization by the DBA mouse metastatic tumor line ESb (42). Moreover, hepatocytes have long been described as possessing asialoglycoprotein receptors involved in the removal from the circulation of desialylated glycoproteins in which galactose residues have become exposed (43). In view of this evidence it is tempting to speculate that galactosyl residues present in abundance on the surface of F9 cells might be involved in liver retention, either through the asialoglycoprotein receptor or via a galactose binding protein of the hepatocyte membrane. Preliminary experiments have shown that F9 cells adhere better to the human hepatoma cell line HepG2, which expresses the asialoglycoprotein receptor (44), compared to F9 cell monolayers (45).

We have also shown that, in spite of the high degree of specificity, the organ colonization properties of F9 cell populations are changed by treatment with RA (Table 2-4). This observation raises some questions that will be considered below. The observations that lung nodule-derived cells are undifferentiated suggests that lung colonies are composed of EC cells. This conclusion is supported by histological evidence. Furthermore, we have shown that RA-treated populations always contain a fraction of cells that retain EC morphology and that these cells increase in number after removal of the inducer. Consequently, it is possible that lung colonies are formed by undifferentiated cells through some interaction with differentiated cells (25). We investigated this possibility by injecting mixtures of treated and untreated populations. If the interaction hypothesis were correct, one would have expected an increase in lung colonization by the mixed population compared to the control population (only treated cells), measurable as an increase in either the number of colonies per lung and/or the number of animals with lung colonies. Our experimental results show that neither of the two parameters change significantly and thus do not support the interaction hypothesis. Small

**DISCUSSION**

The results reported above (Table 1) show that undifferentiated F9 cells have the property to specifically colonize the liver of syngeneic animals. After RA treatment this pattern is drastically changed and the resulting cell population acquires the capacity to colonize the lungs. Single organ specificity has been previously reported for other EC cells. Thus, cells derived from an ovarian teratocarcinoma of strain LT mice (7) and the EC cell line NF-1 of BALB/c mice (9) were found to colonize the ovary. The latter cells also colonized the adrenal gland of syngeneic animals. Cell suspensions from 0TT6050 tumor of strain 129 mice were found to home to the spleen (6) while cells from embryoid bodies of the same tumor colonized the lungs (8). In three of the above cited cases the cells were injected in the lateral tail vein and in the case of ovarian teratoma derived cells, ovary colonization was independent of the route of dissemination (7). The data we present here describe yet another case of organ specificity of colonization by teratocarcinoma cells, namely that F9 EC cells home preferentially to the liver.

The organ colonization pattern observed with systemically inoculated F9 and other EC cells (6, 7, 9) is consistent with the hypothesis of Paget, according to which the formation of metastases depends on an interaction between the tumor cells and the organ colonized rather than being simply a result of the vascular connections between the primary and secondary site (28). Similar conclusions have been reached in a murine system and in the human (29, 30). Furthermore, the fact that our cells acquire the capacity to colonize the lungs only after treatment with RA suggests that only tumor cells endowed with specific properties may succeed in the colonization of the first capillary bed encountered.

**Table 4 Organ distribution of tumors obtained after injection of mixed populations of RA-treated and -untreated cells**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Tumorigenicity</th>
<th>Lung</th>
<th>Liver</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>F9</td>
<td>4/7</td>
<td>1/7 (0, 0, 0, 0, 0, 0)</td>
<td>4/7 (15, 6, 5, 8)</td>
<td>0/7</td>
</tr>
<tr>
<td>F9 + RA + DBC</td>
<td>5/7</td>
<td>5/7 (0, 0, 1, 1, 1, 1, 15)</td>
<td>2/7 (0, 0, 0, 3, 0, 0, 2)</td>
<td>0/7</td>
</tr>
<tr>
<td>F9/F9 + RA + DBC</td>
<td>14/20</td>
<td>13/20 (0, 2, 2, 3, 1, 0, 4, 9, 3, 0, 0, 0, 0, 5, 3, 4, 1, 2, 6, 5)</td>
<td>9/20 (0, 0, 1, 1, 0, 5, 5, 1, 2, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0)</td>
<td>1/20</td>
</tr>
</tbody>
</table>

* Tumorigenicity and organ distribution are defined in Table 1.

Numbers in parenthesis, number of colonies at that site in each animal.

**Fig. 5.** Column chromatography of 3H-Fucose-labeled surface glycopeptides from differentiated (O) and undifferentiated (0) F9 cells. A, G50 elution profiles. *Markers from left to right,* blue dextran 2000 (M, 2,000,000), ovalbumin glycopeptides Man-GlcNAc2-Ang (M, 1670), N-acetylglucosamine (M, 180). B, G100 elution profiles of the G50 excluded peaks. *Markers from left to right,* blue dextran 2000, bovine serum albumin (M, 67,000), chymotrypsinogen A (M, 25,000), ribonuclease (M, 13,700), and galactose (M, 180).

copeptides with differentiation, a shift can also be observed in the relative proportions of the two components. Recently, we found a similar glycopeptide (27) on the surface of two parietal endoderm cell lines PYS-2 and F9ACC19 (21, 22). These results suggest that the presence of the new glycopeptides correlated with differentiation into parietal endoderm.

The cell surface is thought to play an important role in defining the organ specificity phenomena probably involved in the homing of metastatic cells (31, 32) and lymphocytes (33). The cell surface of F9 cells has been extensively studied and it is characterized by the presence of embryonal antigens (34, 35), lectin receptors (36, 37), specific glycoproteins (23), and glycolipids (38). Among the best studied structures of F9 cell surface are the embryoglycans (39) and SSEA antigens (40, 41) also typical of other EC cell lines. Both of these surface components are carbohydrate in nature, containing terminally linked galactose residues (39-41).

Recently a galactose binding protein has been described in mouse liver that could be involved in liver colonization by the DBA mouse metastatic tumor line ESb (42). Moreover, hepatocytes have long been described as possessing asialoglycoprotein receptors involved in the removal of the circulation of desialylated glycoproteins in which galactose residues have become exposed (43). In view of this evidence it is tempting to speculate that galactosyl residues present in abundance on the surface of F9 cells might be involved in liver retention, either through the asialoglycoprotein receptor or via a galactose binding protein of the hepatocyte membrane. Preliminary experiments have shown that F9 cells adhere better to the human hepatoma cell line HepG2, which expresses the asialoglycoprotein receptor (44), compared to F9 cell monolayers (45).
variations, however, might not be detected by this method. Another possibility is that lung nodules derive from a fraction of F9 cells that, although responding to RA, did not become stably differentiated. These cells would thus be modulated to a cell type that did not express SSEA-1 antigens but had acquired some new property that allowed lung arrest and colonization. Once in the lung parenchyma, and in the absence of the inducer, these cells reverted to the original EC morphology but still remained SSEA-1 negative. Thus, modulation of the phenotype rather than differentiation might be responsible for the observed change of organ preference that results after RA treatment. Some of the changes produced during modulation might involve the cell surface (16, 18, 21, 26–28) and this might in turn help to explain the observed change in colonization behavior. Both the above interpretation and the role of cell surface molecules in organ colonization are presently under investigation.

Although the mechanism involved in the shift of organ colonization pattern reported above is not yet completely clarified, the observation that RA induces this change makes our system potentially useful for studies of the molecular bases of organ specific colonization.

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Organ Colonization Pattern of Retinoic Acid-treated and -untreated Mouse Embryonal Carcinoma F9 Cells

Benedetto Terrana, Dario Rusciano and Lorenzo Pacenti


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