Surface Proteins and Fibrinolytic Activity of Cultured Mammalian Cells

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SUMMARY

Surface proteins and fibrinolysis were investigated in a variety of cell types. A large external transformation-sensitive protein was demonstrated by lactoperoxidase-catalyzed iodination. Electron microscope autoradiography confirmed that the technique labeled surface material only. The protein was present in explants of normal tissues was well as in nontumor-producing cultured cell lines. It was not lost during long-term culture. Neoplastic transformation in vitro, whether spontaneous or induced by a chemical carcinogen or virus, led to the loss of the material in most but not all cases. The protein was also absent from many but not all spontaneous and induced tumors of the different types tested. Elevated fibrinolytic activity was demonstrated in a number of normal tissues and non-tumor-producing cell lines. It was also present in most sarcomas but absent from most carcinomas that we have examined, except for a cell line from a well-differentiated bladder tumor. The correlation between absence of large external transformation-sensitive protein and fibrinolysis was examined, and it was found that activation of plasminogen was not sufficient to cause the absence of this surface protein.

INTRODUCTION

Many criteria exist that investigators have used to differentiate normal from "transformed" cells in vitro. Examples include density-dependent inhibition of growth (34), growth in agar (22), growth dependence on serum concentration (33), cyclic adenosine 3',5'-monophosphate levels during various stages of growth (26); and morphology in culture (24). However, none shows an absolute correlation with true neoplastic transformation. Recently, 2 further changes associated with transformation have been described. The 1st of these is a quantitative increase in cell protease production after transformation (37). This increase in protease activity is mediated by a cell factor (36) that enzymatically activates the serum proenzyme plasminogen to its active form plasmin (28). The plasmin is detected by assaying its activity on a 125I-labeled fibrinogen substrate (37). The 2nd change is the loss of a high-molecular-weight plasma membrane protein, usually identifiable in normal cells after iodination catalyzed by lactoperoxidase. Described by several groups (13-15, 38, 39), this LETSP3 has a molecular weight of 200 to 250 x 10^3 daltons, is a glycoprotein (17, 27), and is not collagen or mucopolysaccharide (17). Its presence at the surface is temperature sensitive in cells transformed by ts mutants of oncogenic viruses (13, 19, 20, 31, 35, 38, 39).

Most of the criteria for neoplastic transformation in vitro have been studied using long-established cell lines transformed by oncogenic viruses. We have studied these types of cell lines as well as a series of cell lines established from normal tissues and from spontaneous and induced tumors of several species. These included 2 lines maintained for some years in a completely synthetic protein-free medium. Primary cell suspensions, primary cultures, and cultures of normal tissue at different transfer generations were also used to control changes that may have been induced by culture conditions. We have investigated fibrinolytic activity and the presence or absence of LETSP in these various cell types, primarily to investigate whether the 2 might be related.

MATERIALS AND METHODS

Cells. Cells (see references in Tables 1 and 2) were maintained in Dulbecco's modification of Eagle's medium or Waymouth's Medium 752/1 supplemented with 10% calf serum. All spontaneously and chemically transformed cells listed in Table 2 have produced histologically confirmed mesenchymal tumors after inoculation into syngeneic hosts (Refs. 9 and 40; L. M. Franks, A. W. Carbonell, and V. J. Hemmings, unpublished observations). The virus-transformed cells have the usual criteria of viral transformation growth in agar, transformed cell morphology, and lack of contact inhibition (23, 41). Cells from the spontaneous or induced rodent tumors have produced histologically confirmed tumors similar to the tumor of origin after implantation into syngeneic hosts (L. M. Franks, unpublished observations). Of the human lines, HT 29 (8) was derived from a human colon cancer and retains the ultrastructural features of the tumor from which it arose (L. M. Franks, unpublished observation). The bladder lines RT 4 (30) and T 24 (8) retain the ultrastructural features of bladder tumors (L. M. Franks, unpublished observation). RT 4 cells have produced tumors of differentiated bladder cancer structure after inoculation into immunosuppressed hamsters (30). Line J 82 is an undifferentiated tumor cell line but is almost certainly epithelial.

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3 The abbreviation used is: LETSP, large external transformation-sensitive protein.

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since it was established from a bladder carcinoma and spontaneously transformed human mesenchymal cell lines have not been established.

Lactoperoxidase-catalyzed Iodination. Cells were iodinated directly on the dish as previously described (15, 27).

Polyacrylamide Gel Electrophoresis. This was performed in buffers containing sodium dodecyl sulfate as previously described (15, 27). Gels were dried in a vacuum and autoradiographed to locate labeled proteins. The amount of LETSP present was estimated relative to the other labeled proteins as follows: +++, strong labeling of LETSP comprising 20 to 50% of total radioactivity; ++, moderate labeling comprising 10 to 20% of total radioactivity; +, weak labeling, with LETSP barely detectable and representing 1 to 5% of the total radioactivity; --, no detectable radioactivity in LETSP region, representing less than 1% of the total radioactivity.

Fibrinolytic Assay. Purified fibrinogen (21) was labeled with [125I]-iodide using lactoperoxidase. The fibrin dish assay was performed as previously described (37) except that 20 μg fibrinogen per sq cm were used; 25,000 cpm were plated onto each dish. Prior to plating onto the fibrinogen-covered dishes, cells were washed twice with serum-free medium, gently scraped off the dish, and resuspended by pipetting. Cell number was determined by Coulter counting (Coulter Electronics Ltd., Hertfordshire, England) and 10⁶ cells were plated on the fibrinogen-coated dish. When no cells were added to the fibrinogen dish, less than 5% of the radioactivity was solubilized. Cells were cultured for 24 hr in the presence of 1% calf serum previously inactivated at pH 3 for 2 hr at room temperature (25). Medium was removed at that time and counted directly in a well-type γ-scintillation counter (Nuclear Enterprises Inc., San Carlos, Calif.).

Electron Microscopy. Cells were fixed directly on the dish, after iodination, in 2.5% glutaraldehyde, postfixed in Palade’s fluid, and embedded in Araldite. Sections were prepared for autoradiography using Ilford L4 emulsion, and exposed for periods varying from 2 to 9 weeks.

RESULTS

Fig. 1 shows an autoradiograph after electrophoresis on sodium dodecyl sulfate-polyacrylamide gels of cells iodinated by lactoperoxidase. These representative gel patterns demonstrate the variation found in LETSP expression on the cell surface. Fig. 1 shows that 2 strains of normal human embryo diploid lung cells (Fig. 1, A and B) both contained ++ + levels of LETSP. It was retained at this level through at least 24 transfer generations in culture (data not shown). Also, 3 cell lines derived from mouse sarcomas contained detectable amounts of LETSP (Fig. 1, C, E, and F). The spontaneous mouse sarcoma, CMT 81 (Fig. 1 C) contains +++ levels of LETSP while the remaining 2 sarcoma lines (Fig. 1, E and F) contain ++ + levels. The human carcinoma, HT 29 (Fig. 1 D) was negative. Electron microscope autoradiographs (Fig. 2) of labeled cells confirmed that the lactoperoxidase-labeling technique labels only plasma membrane-associated proteins. Internal organelles were not iodinated.

Table 1 summarizes the data obtained when normal cells were assayed for LETSP and fibrinolytic activity. It can be seen that, regardless of whether the cells were obtained from established cell lines or were recent isolates, they all contained detectable amounts of LETSP. Cells isolated from calf bladder and human prostate and normal embryo diploid cells of lung (5 strains examined) hydrolyzed between 60 and 100% of the radioactive fibrin after 24 hr. The remaining cell lines hydrolyzed less than 25% of the radioactive fibrin under the same conditions.

Table 2 shows the results obtained with several lines of spontaneously, chemically, or virally transformed cells. Several of the sarcoma cells tested here contained LETSP, whereas all the carcinomas examined were negative. Of the sarcoma cell lines positive for LETSP, 3 were derived from the 1 parent type, COM 5/T, and many represent some peculiarity of the cell type. Nevertheless, these cells were tumor producing in vivo (11). With the exception of Py 3T3, all virally transformed sarcoma-producing cell lines were negative for LETSP in agreement with earlier results (13–15, 17, 19, 20, 27, 31, 35, 38, 39). For convenience, LX cells have been listed as sarcoma producing although this has not been firmly established (P. N. Riddle, unpublished observation).

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Table 1

Normal cells assayed by iodination and for fibrinolytic activity

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue</th>
<th>Cell lines</th>
<th>LETSPa</th>
<th>Fibrinolytic activity (% cpm in medium)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary explant</td>
<td>Human</td>
<td>Prostate</td>
<td>+++</td>
<td>79</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Calf</td>
<td>Bladder</td>
<td>++++</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rat</td>
<td>Mammary</td>
<td>+++</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Whole embryo</td>
<td>Mouse</td>
<td>CME7/2</td>
<td>++++</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Diploid cells</td>
<td>Human</td>
<td>Lung</td>
<td>HE75</td>
<td>60</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HE88</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HE81</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HE31</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>Fibroblast lines</td>
<td>Mouse</td>
<td>HE33</td>
<td>+++</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hamster</td>
<td>HE31</td>
<td>+++</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HE75</td>
<td>++++</td>
<td>88</td>
<td></td>
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<td></td>
<td></td>
<td>HE88</td>
<td>++++</td>
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<td>HE81</td>
<td>+++</td>
<td>16</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>HE31</td>
<td>+++</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

a LETSP quantitated as described in "Materials and Methods."

Table 2

Transformed cells assayed by iodination and for fibrinolytic activity

<table>
<thead>
<tr>
<th>Sarcomas</th>
<th>Species</th>
<th>Tissue</th>
<th>Cell lines</th>
<th>LETSPa</th>
<th>Fibrinolytic activity (% cpm in media)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Transformed&quot; in vitro</td>
<td>Mouse</td>
<td>Tongue</td>
<td>COM 5/T</td>
<td>++</td>
<td>41</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>COM 5/TX</td>
<td>++</td>
<td>24</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Salivary gland</td>
<td>CSG 46</td>
<td>-</td>
<td>55</td>
<td>40</td>
</tr>
<tr>
<td>Viral</td>
<td>Mouse</td>
<td>SV 3T3</td>
<td>-</td>
<td>90</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Py 3T3</td>
<td>++</td>
<td>100</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hamster</td>
<td>Py BHK</td>
<td>-</td>
<td>100</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HSV NIL8</td>
<td>-</td>
<td>100</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Carcinogen</td>
<td>(9,10-Dimethyl-1,2-benzanthracene) mouse</td>
<td>Salivary gland</td>
<td>CSG 58</td>
<td>-</td>
<td>56</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>(20-Methylcholanthrene) mouse</td>
<td>L</td>
<td>-</td>
<td>NTa</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>From transformed cell lines</td>
<td>Mouse</td>
<td>From L-cells</td>
<td>LX</td>
<td>+++</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>Spontaneous</td>
<td>Mouse</td>
<td>From COM 5/TX</td>
<td>CMT 80</td>
<td>++</td>
<td>43</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>s.c.</td>
<td>CMT 81</td>
<td>++++</td>
<td>100</td>
<td>32b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>s.c.</td>
<td>CMT 82</td>
<td>+</td>
<td>19</td>
<td>32b</td>
<td></td>
</tr>
<tr>
<td>Carcinomas</td>
<td>Mouse</td>
<td>Lung</td>
<td>CMT 64</td>
<td>-</td>
<td>28</td>
<td>32c</td>
</tr>
<tr>
<td>Spontaneous</td>
<td>Mouse</td>
<td>Lung</td>
<td>CMT 68</td>
<td>-</td>
<td>21</td>
<td>32c</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>Mammary</td>
<td>CMT 67</td>
<td>-</td>
<td>26</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td>Colon</td>
<td>HT 29</td>
<td>-</td>
<td>15</td>
<td>8</td>
<td>d</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>RT 4</td>
<td>-</td>
<td>90</td>
<td>30</td>
<td>d</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>J 82</td>
<td>-</td>
<td>24</td>
<td>1</td>
<td>d</td>
</tr>
<tr>
<td></td>
<td>Bladder</td>
<td>T 24</td>
<td>-</td>
<td>17</td>
<td>4</td>
<td>e</td>
</tr>
<tr>
<td></td>
<td>Mammary</td>
<td>-</td>
<td></td>
<td>100</td>
<td>r</td>
<td></td>
</tr>
</tbody>
</table>

a LETSP quantitated as described in "Materials and Methods."
b NT, not tested.
c L. M. Franks and V. J. Hemmings, in preparation. Culture methods as in Ref. 11.
d C. O'Toole, unpublished observations.
e R. C. Hallowes, unpublished observations.

The distribution of fibrinolytic activity among the transformed cells (Table 2) was as follows. Virally transformed cells solubilized 90 to 100% of the radioactive fibrin after 24 hr. Of 8 carcinoma cell lines tested, 2 solubilized greater than 90% of the fibrin; the remaining 6 solubilized less than 28%. The distribution of fibrinolytic activity among the 11 sarcoma-producing cell lines was more varied, with 2 lines solubilizing less than 24% of the precipitated fibrin and the remaining 9 cell lines hydrolyzing 41 to 100%. Thus, sarcoma-producing cell lines were much more likely to produce fibrinolytic activity than were those derived from carcinomas. The use of calf serum previously treated at low pH to inactivate inhibitors (25) makes it unlikely that other sera would allow fibrinolysis, which was not detected in these experiments. However, this possibility cannot be rigorously excluded.
DISCUSSION

The results obtained with this limited sample of normal and transformed cells, together with previous data (13–15, 17, 19, 20, 27, 31, 35, 38, 39), indicate that LETSP is present on many normal cells from a wide variety of species but is absent from most transformed or tumor cells. Increased levels of fibrinolytic activity are present in some tumor cells (Table 2), mainly sarcomas, but are also present in some normal cells (Table 1). In considering the use of these 2 parameters in identifying transformed cells, neither criterion is absolute since exceptions to both exist. Most of the normal cells examined were fibroblastic and, in agreement with earlier reports, these all had high levels of LETSP on their surfaces. The primary explants of epithelial tissues that we examined were positive for this protein, although it is not clear whether or not this was due to fibroblast contamination.

After 24 hr in culture under the conditions described, cell lines BHK and NIL 8 hydrolyzed 25% of the radioactive fibrin. These cell lines are widely accepted as being representative of normal cell types as judged by many criteria, including growth in agar (22), density-dependent inhibition of growth (34), and morphology in culture (24). Thus, a level of approximately 30% solubilization of radioactivity represents the maximal fibrinolytic activity of low-producer normal cells. This level may vary depending on the amount of fibrin precipitated on the dish, the serum used, and number of cells plated; and care must be taken to standardize conditions and always to include normal cell type controls for comparative purposes.

Several cell lines exhibit 100% solubilization of radioactive fibrin before 24 hr. Although they vary in the kinetics of release of labeled material, within the context of these experiments all have been classified as active producers of fibrinolysis.

The observation that several types of normal cells, in particular lung and bladder, produce substantial amounts of fibrinolytic activity is consistent with reports by other investigators (2, 3, 39). It is not clear how high levels of LETSP persist on the surfaces of lung fibroblasts that are active in fibrinolysis, since this protein is known to be sensitive to proteases, including plasmin in the case of hamster NIL 8 fibroblasts (20). However, this result indicates that activation of plasminogen by plasminogen activator were involved in this process, one would predict that cells positive for fibrinolysis would be negative for LETSP. However, as mentioned earlier, normal cells exist that are positive for both parameters (Table 1), and this is also true for several transformed cell lines (see Table 2). These results show that activation of plasminogen is not sufficient for loss of LETSP. Results reported elsewhere (18, 20) suggest that it is not necessary. However, none of the data precludes the involvement of another protease different from the plasminogen activator (cf. Ref. 5). Further work will be required to investigate this possibility. Absence of this protein from cell surfaces could also be caused by mechanisms other than proteolysis.

The results of iodination of the transformed cells show that viral transformation leads to a loss or marked reduction in levels of LETSP at the cell surface. The Py 3T3 tested here was an exception, in conflict with earlier results (14), and this cell line has not been tested for presence of the transforming virus. We have reported earlier (15) that Py NIL 8 cells retain a small amount of LETSP on their surfaces. Thus the loss is not always complete, although in most cases all-or-nothing effects have been observed (13–15, 18, 27, 38, 39).

Chemically transformed rat fibroblasts have also been reported to lose LETSP completely (6), and the carcinogen-induced sarcoma and carcinoma reported here were also negative (Table 2).

The spontaneously transformed and tumor-derived lines studied here gave less uniform results. All the carcinomas were negative, whereas several of the sarcomas were positive for LETSP. The latter class included a series of cells derived from a single original isolate, COM5/T, and the serum-independent line, LX, previously reported to be positive (14, 15). LX is not known to be malignant, but several of the other lines are. While these exceptions show that loss of LETSP is not required for malignancy, they should be considered in the context of the large number of induced transformed cells, where loss of this protein correlates with transformation, including cases of temperature-sensitive expression of transformation (13, 19, 20, 31, 35, 38, 39).

LETSP is known to be susceptible to removal from the cell surface by low levels of protease (15, 18–20, 27). Since transformed cells produce high levels of a protease, plasminogen activator (28, 36, 37), it seemed possible that the loss of this protein from transformed cell surfaces might involve an autoproteolytic mechanism (16). If release of plasminogen activator were involved in this process, one would predict that cells positive for fibrinolysis would be negative for LETSP. However, as mentioned earlier, normal cells exist that are positive for both parameters (Table 1), and this is also true for several transformed cell lines (see Table 2). These results show that activation of plasminogen is not sufficient for loss of LETSP. Results reported elsewhere (18, 20) suggest that it is not necessary. However, none of the data precludes the involvement of another protease different from the plasminogen activator (cf. Ref. 5). Further work will be required to investigate this possibility. Absence of this protein from cell surfaces could also be caused by mechanisms other than proteolysis.

REFERENCES

6. Clarke, S. M., Simpson, V. J., Benedict, W. F., and Fink, L. M. Surface


Fig. 2. Electron microscopic autoradiographs of iodinated mouse sarcoma cells (CMT 80). Glutaraldehyde and osmic acid fixation, Ilford L4 emulsion exposed for 63 days, stained with lead citrate. Detail of surface labeling (x 30,000) from group of cells shown in inset (x 4000). There is no cytoplasmic labeling.
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