Growth Factors Produced by Sarcoma Virus-transformed Cells

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Abstract

A hypothesis is presented that states that tumor viruses produce their transforming action on cells, at least in part, by the production of endogenous polypeptide growth-stimulatory factors. Cells transformed by murine sarcoma viruses have been found to have reduced or absent cell surface receptors for epidermal growth factor (EGF), as compared to the untransformed parental cells and to cells transformed by other viruses. Sarcoma virus-transformed cells are shown to release a family of polypeptide growth factors into the supernatant fluids in cell cultures. These factors, with molecular weights of approximately 25,000, 12,000, and 7,000, stimulate cell division and compete for EGF receptors. The new sarcoma growth factors are not produced by untransformed cells or by DNA virus-transformed cells. Sarcoma growth factors induce normal fibroblasts to grow in soft agar and to express some of the phenotypic properties of transformed cells; these effects are dependent on the continued presence of the factor.

A human fibrosarcoma was found that had normal levels of EGF receptors, but no apparent multiplication-stimulating activity (MSA) receptors. Since normal fibroblasts have MSA receptors, it was decided to test whether the receptor-negative cell was producing a related growth factor. In this case a family of related growth polypeptides was found that interacted with MSA receptors, but not with EGF receptors, and stimulated cell division in cells of various species. The activities of both the sarcoma growth factors and the MSA-related human fibrosarcoma growth factors are heat stable, protease sensitive, and sensitive to disulfide reducing agents. The endogenous production of polypeptide growth factors by cells that are able to respond to their own products may represent a general mechanism for cell transformation.

Introduction

Over the years our laboratory and many other laboratories have found that cells in culture can be transformed by a variety of different agents such as DNA viruses and RNA viruses (both mammalian and avian), as well as by chemical carcinogens and radiation. Two widely used cell systems for the study of transformation are the mouse cell lines, random bred Swiss 3T3 (25) and BALB/3T3 (1). We have been interested in those properties common to transformed cells that distinguish them from normal cells and also in the characteristic properties conferred by particular transforming agents. For example, cells transformed by the DNA tumor viruses, SV40 or polyoma, contain a characteristic nuclear antigen called T-antigen (3, 12, 26). In addition they contain a smaller T-antigen in the cytoplasm that has some antigenic determinants in common with the larger nuclear T-antigen (21). These new viral-coded proteins, then, serve as markers that identify the specific transforming virus and, perhaps, are themselves involved in the transformation process (29).

Approximately 2 years ago, we noted that mouse, rat, or mink cells transformed by MSV² characteristically lost their cell surface receptors which bind the polypeptide growth promoter EGF. The sarcoma virus-transformed cells show a dramatic decrease or total absence of cell surface EGF receptors, as determined by their binding of ¹²⁵I-labeled EGF (23). This, however, is not a general phenomenon of transformation, since neither the DNA virus-transformed cells nor the majority of chemically transformed cells showed this characteristic loss of available EGF receptors (23, 24). The change was relatively rapid, detectable within days after infection of a culture with high-titered MSV. One explanation for the loss of available EGF receptors following sarcoma virus transformation is that the cells themselves produce a substance capable of interacting with these receptors. The possibility was suggested that a product of the MSV-transformed cells blocks the EGF receptors and might also serve as an endogenous growth stimulator that is either directly produced by the viral genomes or is a cellular gene product expressed in mammalian sarcoma virus-transformed cells (23, 24).

Sarcoma Virus and EGF Receptors

The murine sarcoma virus has basically 3 genes that are required for replication (2). As summarized in a simplified form in Table 1, these are the env gene, the gag gene, and the pol gene. Each is essential for replication of type C viruses, but they do not appear to be involved in the process of transforming normal fibroblasts into fibrosarcoma cells. There is also a fourth gene that is essential for transformation, but is not essential for viral growth. This has been called onc or sar and has been most thoroughly studied with the avian sarcoma viruses, in which deletion mutants that lack the transforming function are available (19, 20). Sarcoma viruses may arise by recombination between viral genes and host cellular genes. Some of the properties that would be expected of a potential transforming gene are listed, based on what is known of the sarcoma-specific nucleic acid sequences, derived primarily from studies with deletion mutants of the avian sarcoma viruses: (a) present in normal cellular DNA; (b) well-conserved in evolution; (c) codes for protein (maximum M.W. 60,000); (d) not essential

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² The abbreviations used are: MSV, mouse sarcoma virus; EGF, epidermal growth factor; MSA, multiplication-stimulating activity; SGF, sarcoma growth factor.

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for virus replication; and (e) product acts as a regulator of cell growth. It is evident that one of the classes of proteins that could satisfy the requirements for transforming proteins would be normal or abnormal forms of the polypeptide growth hormones, such as EGF, fibroblast growth factor, or the insulin-like growth factors (5, 11, 16). The specific loss of EGF receptors from MSV-transformed cells was the first indication that there was in fact an association between viral transformation and the expression of available receptors for specific growth factors on these cells.

Table 2 shows typical data on a variety of different transformed mouse 3T3 cells. The EGF binding capacity of the majority of these cell lines is not altered from the levels expressed by the untransformed controls. Only the binding levels of mouse sarcoma virus-transformed cells are affected. SV40 and polyoma virus-transformed cells, as well as avian sarcoma virus transformants, showed normal levels of EGF receptors comparable to those of the controls. Actual virus production did not affect EGF binding, although it did specifically block binding of the 125I-labeled env gene product glycoprotein 71 (7). In contrast, MSA, a growth factor which is produced by rat liver cells (10, 15), as avian sarcoma virus transformants, showed normal levels of mouse sarcoma virus-transformed cells are affected. SV40 and polyoma virus-transformed cells, as well as avian sarcoma virus transformants, showed normal levels of EGF receptors comparable to those of the controls. Actual virus production did not affect EGF binding, although it did specifically block binding of the 125I-labeled env gene product glycoprotein 71 (7). In contrast, MSA, a growth factor which is produced by rat liver cells (10, 15), as avian sarcoma virus transformants, showed normal levels of mouse sarcoma virus-transformed cells as well as, or slightly better than, to the untransformed cells (Table 3). In addition, the loss of receptors resulting from MSV transformation is evident in mouse, rat, and mink cells transformed by MSV and in hamster cells transformed by the Harvey strain of MSV; in none of the cases was MSA binding reduced.

The results comparing various mouse cells transformed by a variety of agents are summarized in Table 4. None of the SV40- or polyoma-transformed clones had diminished EGF receptors. Only a small fraction (5 out of 47) of the chemically transformed cells had lost their EGF-specific receptors; these included one clone of benzo-pyrene-transformed BALB/3T3, one clone of dimethylbenz(a)anthracene-transformed mouse BALB/3T3 cells, and a C3H/10T1/2 cell clone transformed by methylcholanthrene. Loss of EGF receptors after transformation, then, seemed to be specifically associated with sarcoma virus transformation, although a subset of chemically transformed cells also showed this phenotype.
EGF and MSA receptors in early passages in cell culture, as well as at the end of their in vitro lifetime. Embryonic, newborn, and aged adult cells all had receptors, as did cells transformed by the DNA tumor virus SV40 (28). Among the tumor cells there were 2 striking exceptions for EGF binding, a human rhabdomyosarcoma and a bronchogenic carcinoma. We have not as yet followed up these cells to determine whether they fail to bind EGF because of endogenous production of a related growth factor. Similarly, the great majority of normal and tumor cell lines have MSA receptors. Among the tumor cells, 2 lines stand out for having especially high levels of MSA receptors. One of these is a glioblastoma, A1723, and the other is a chondrosarcoma, A1684. The chondrosarcoma is of interest because cartilage cells were the cells used to first define the class of somatomedin-like growth factors (6) that include rat MSA. It was significant that the 2 fibrosarcoma lines tested were negative for MSA binding, given that over 40 human diploid fibroblast strains have been tested and all have MSA receptors. One of the fibrosarcomas, line 8387 (8), was chosen for study in greater detail. Chart 1 shows the time course of EGF and MSA binding to sarcoma virus-transformed cells, as compared to that of normal cells, and to human fibrosarcoma cells, as compared to their normal fibroblast counterparts. Normal fibroblasts have receptors for both the EGF and the MSA family of growth polypeptides. They can also respond by cell DNA synthesis to either or both of these factors (data not shown). Sarcoma virus-transformed cells, whether they are derived from mouse, rat, or mink, have a decreased number, or

### Table 4

<table>
<thead>
<tr>
<th>Cells</th>
<th>Clones with decreased EGF binding</th>
<th>% Clones with decreased EGF binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untransformed parental clones</td>
<td>0/35</td>
<td>0</td>
</tr>
<tr>
<td>SV40 transformed</td>
<td>0/8</td>
<td>0</td>
</tr>
<tr>
<td>Polyoma transformed</td>
<td>0/4</td>
<td>0</td>
</tr>
<tr>
<td>MSV transformed</td>
<td>12/12</td>
<td>100</td>
</tr>
<tr>
<td>Chemically transformed</td>
<td>5/47</td>
<td>11</td>
</tr>
</tbody>
</table>

### Table 5

<table>
<thead>
<tr>
<th>Normal fibroblasts</th>
<th>125I-labeled MSA binding in human cells (cpm/10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic lung</td>
<td>7,200</td>
</tr>
<tr>
<td>Newborn foreskin</td>
<td>5,160</td>
</tr>
<tr>
<td>Adult skin</td>
<td>5,800</td>
</tr>
<tr>
<td>Adult skin (SV40 transformed)</td>
<td>7,800</td>
</tr>
<tr>
<td>Tumor cell lines</td>
<td></td>
</tr>
<tr>
<td>Chondrosarcoma</td>
<td>23,600</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>19,400</td>
</tr>
<tr>
<td>Renal cell carcinoma</td>
<td>4,800</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>&lt;50</td>
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</table>

### Chart 1

**Time course of association of 125I-labeled EGF (a,c) and 125I-labeled MSA (b,d) to different cell types.** In a and b, 125I-labeled EGF (a) or 125I-labeled MSA (b) were incubated with normal mink cells (●, 3 x 10⁵ cells/dish) or Kirsten sarcoma virus-transformed mink cells (○, 6 x 10⁵ cells/dish). In c and d, 125I-labeled EGF (c) or 125I-labeled MSA (d) were incubated with 4 human lines or strains: 8387, a human fibrosarcoma line (●, 3 x 10⁵ cells/dish); HT 1080, a human fibrosarcoma line (○, 5 x 10⁵ cells/dish); Huf, a human foreskin strain (□, 4 x 10⁶ cells/dish); and WI38, a human embryonic lung fibroblast strain (●, 4 x 10⁶ cells/dish). The cpm bound are plotted against the time of incubation (min). The data have been corrected for neither counter background (∼60 cpm) nor nonspecific binding (∼250 cpm for 125I-labeled MSA). Reproduced from Todaro et al. (24).
total loss of, EGF receptors, while the levels of available MSA receptors are either unchanged or slightly increased. The converse situation exists for the human fibrosarcomas; they have lost their MSA receptors, but maintained normal or even elevated levels of measurable EGF receptors. One way to explain these data is to postulate that MSV-transformed cells make an EGF-related growth factor capable of interacting with the EGF receptor system. This factor(s), then, may serve as an endogenous stimulus to cell growth. The human fibrosarcomas, on the other hand, produce a substance related to MSA that is capable of interacting with the MSA-specific receptors and serving as a signal for cell division (Chart 2). In fact, in both instances this has turned out to be the case.

Sarcoma Cell-produced Growth Factors

We will describe the growth factors produced by the human fibrosarcoma cells first and then the growth factors produced by the sarcoma virus-transformed cells. In both cases the starting materials are supernatant fluids from cell cultures maintained on serum-free medium. They are concentrated, usually from 1 to 4 liters of fluid, by dialyzing against 1% acetic acid and lyophilizing. The lyophilized material is extracted with 1 M acetic acid and run over a sizing column of either Sephadex G-75 or Bio-Gel P-60. The fractions are assayed for EGF and MSA competing activity. Chart 3 shows the peak fractions, separated by a column of Sephadex G-75 superfine (1.5 x 90 cm), to give the data shown. The protein markers were ovalbumin (45,000), carbonic anhydrase (31,000), myoglobin (16,900), RNase (13,800), and insulin (6,000). The competing activity in the G-75 fractions was determined with the use of a radioreceptor assay on subconfluent monolayers of cell cultures. Approximately 5 x 10⁵ normal rat kidney cells were seeded per 35-mm plate well (Costar 3506). The cells were seeded in Dulbecco's modified Eagle's medium containing 10% calf serum 24 hr before binding ¹²⁵I-labeled MSA. The cells were washed twice with 2-ml portions of binding buffer, which consists of Dulbecco's modified Eagle's medium containing bovine serum albumin (1 mg/ml) and 50 mM N,N-bis(2-hydroxyethyl)2-aminoethanesulfonic acid, adjusted to pH 6.8. Binding reactions were initiated by the addition of 1 ml of binding buffer to remove the unbound ¹²⁵I-labeled MSA. The amount of radioactivity bound to the cells was measured after lysing of the cells. Radioactivity present in the lysate was determined with the use of a Beckman 7000 γ counter at 67% efficiency. Nonspecific binding was estimated by determining the amount of cell-bound radioactivity in the presence of 1 μg of labeled MSA. Under these conditions, the cells specifically bound 2.5% of the input counts or 405 cpm above the nonspecific binding (75 cpm). O.A., ovalbumin; C.A., carbonic anhydrase; MyO, myoglobin; Ins., insulin. Reproduced from De Larco and Todaro (8).

Chart 2. Model showing normal fibroblasts with specific receptors for EGF and for MSA binding and the altered cell phenotype displayed by MSV-transformed cells and by human fibrosarcoma cell lines in culture.

Chart 3. MSA competing activity produced by human fibrosarcoma cells. A confluent monolayer of human fibrosarcoma cells was incubated with serum-free Waymouth's tissue culture medium (13) for 48 hr, the medium was clarified of cellular debris by centrifugation, and the supernatant fluids were used as the conditioned medium. The MSA competing activity was initially fractionated on a column of Dowex 50 resin (10). The competing activity from this column was further characterized on a column of Sephadex G-150 (5 x 90 cm) which was developed and eluted with 1 M acetic acid. This column gave 4 broad peaks, designated I through IV, of MSA competing activity as measured by the radioreceptor assay. Center cuts were taken from each of these peaks and rechromatographed on a second acid-gel filtration column, Sephadex G-75 superfine (1.5 x 90 cm), to give the data shown. The protein markers were ovalbumin (45,000), carbonic anhydrase (31,000), myoglobin (16,900), RNase (13,800), and insulin (6,000). The competing activity in the G-75 fractions was determined with the use of a radioreceptor assay on subconfluent monolayers of cell cultures. Approximately 5 x 10⁵ normal rat kidney cells were seeded per 35-mm plate well (Costar 3506). The cells were seeded in Dulbecco's modified Eagle's medium containing 10% calf serum 24 hr before binding ¹²⁵I-labeled MSA. The cells were washed twice with 2 ml portions of binding buffer, which consists of Dulbecco's modified Eagle's medium containing bovine serum albumin (1 mg/ml) and 50 mM N,N-bis(2-hydroxyethyl)2-aminoethanesulfonic acid, adjusted to pH 6.8. Binding reactions were initiated by the addition of 1 ml of binding buffer containing 1 ng of ¹²⁵I-labeled MSA (11,600 cpm) by itself or in the presence of unlabeled MSA or material from a column fraction. The plates were incubated at 22° for 90 min. After incubation, the medium was removed, and the cell monolayers were washed 4 times with cold binding buffer to remove the unbound ¹²⁵I-labeled MSA. The amount of radiolabeled MSA bound to the cells was measured after lysing of the cells. Radioactivity present in the lysate was determined with the use of a Beckman 7000 γ counter at 67% efficiency. Nonspecific binding was estimated by determining the amount of cell-bound radioactivity in the presence of 1 μg of labeled MSA. Under these conditions, the cells specifically bound 2.5% of the input counts or 405 cpm above the nonspecific binding (75 cpm). O.A., ovalbumin; C.A., carbonic anhydrase; MyO, myoglobin; Ins., insulin. Reproduced from De Larco and Todaro (8).
lected for MSA competing activity and passed again over a G-75 column. The largest is a binding protein, rather than a growth-stimulating protein, capable of binding labeled MSA, but itself having no growth-stimulating activity. The other 3, having approximate molecular weights of 20,000, 11,000, and 7,000, each show growth-stimulating activity, with the smallest product being the most active (see Chart 4). The test cells are normal rat kidney fibroblasts. The activities are heat stable, protease sensitive, and sensitive to reducing agents. When tested for growth-stimulating activity and for induction of DNA synthesis in rat fibroblasts, Fraction IV, the 7,000-molecular-weight peak, showed 7-fold stimulation at 20 µg/ml. The material has been further purified on CM-cellulose and is active in nanogram quantities.

Conditioned medium from the sarcoma virus-transformed cells was prepared and tested similarly. The EGF competing activities coeluted with the thymidine-incorporating activity and also with the activity that permits cells to grow in soft agar (Chart 5). On Bio-Gel P-60, 3 peaks are seen. There is a broad, higher-molecular-weight activity between 20,000 and 27,000, then a sharp peak at 12,000 and another at around 7,000.

The ability to induce cells to grow in soft agar conferred by the growth factor present in partially purified supernatant fluids from sarcoma virus-transformed cells, which we are calling SGF, is distinctly different from the properties of other growth factors, such as EGF, fibroblast growth factor, MSA, and the somatomedins (9). None of these other growth factors have the ability to induce normal, anchorage-dependent cells to behave as anchorage-independent cells when tested in a soft agar growth assay. This property, which correlates best with tumorigenicity, is characteristic of sarcoma virus-transformed cells. The transformed cells produce a factor, or series of factors, that confers this anchorage-independent growth ability upon untransformed cells treated with the factors. The active factors are stable to boiling for 3 min, are completely trypsin sensitive, and are destroyed by disulfide reducing agents. The activity can be expressed as agar colony forming units for fibroblasts. The 12,000-dalton peak fraction shows 5 x 10⁴ agar growth units/µg of protein. The larger-molecular-weight region is more heterodisperse; in fact, 2 peaks, one having a molecular weight of approximately 26,000 and the other approximately 20,000, have been resolved. The active 12,000-molecular-weight-fraction represents a several hundred-fold purification over the original starting material (as shown in Chart 6) whether measured by agar growth stimulation, induction of DNA synthesis, or EGF receptor competition.

The ability of SGF to induce cell growth in soft agar is entirely reversible. When soft agar colonies formed by untransformed fibroblasts, under the stimulation of SGF, are selected and plated as monolayer cultures in the absence of SGF, they grow as normal contact-inhibited, growth-arrested fibroblasts. They will not form soft agar colonies when plated in the absence of SGF; however, if SGF is added as an overlay one week after these cells are plated in soft agar, they will form 3 or 4 cell colonies within 3 days and eventually grow to uniform colonies of greater than 100 cells. While MSV produces a permanent transfor-
transformation" including altered morphology, ability to form a colony in soft agar. The clarified, heat-inactivated, conditioned medium (A) has an agar growth unit activity of 4.9 x 10^3/mg, the dialyzed lyophilized concentrate (A) has an agar growth unit activity of 8.7 x 10^4/mg, and Fraction 67 from Chart 6 (O) has an agar growth unit activity of 2.3 x 10^5/mg.

The viruses could, rather, act by allowing the expression of viral genes also resides in the cellular DNA. Viral genes may as the genetic information for making whole virogenes are capable of responding to this product. Therefore, just endogenous growth factors by cells such as fibroblasts that then, would have acquired either the structural or regulatory proteins also resides in the cellular DNA. Viral genes may contain this information or could recombine with it in such a way as to be able to transmit it from cell to cell.

References

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Fig. 1. Normal rat kidney cells. A, untreated; B, treated with an aliquot of SGF (Fraction 47 from Chart 6) at 10 μg/ml and photographed 6 days later. The cells have grown to considerably higher cell density and display a morphology similar to that of viral transformed cells. x 125. In C and D, normal rat kidney cells were plated in 0.3% soft agar. C, untreated. D, Treated with an aliquot of SGF (Fraction 67 from Chart 6) at 10 μg/ml and photographed 2 weeks after treatment. The untreated cultures show primarily single cells with 2 or 3 cell colonies, but none of larger size. In the treated cultures many colonies contained well over 500 cells. x 250.
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