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Transforming growth factor(s) production enables cells to grow in the absence of serum: An autocrine system

(serum-free growth/Kirsten murine sarcoma virus/cell transformation)

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ABSTRACT **Kirsten murine sarcoma virus (KiMSV)-transformed rat-1, normal rat kidney (NRK), and BALB/c 3T3 cells are capable of continual growth in a serum-free medium supplemented with transferrin and insulin but with no exogenous mitogenic growth factors. Cells transformed by a mutant of KiMSV that is temperature sensitive for the maintenance of transformation grow in this medium at the permissive temperature only. At the nonpermissive temperature, growth is dependent upon the presence of serum-free conditioned medium from the transformed cells. Normal rat-1 cells are also dependent upon factors from the transformed cells for growth in this serum-free/mitogen-free medium. The serum-derived growth factors, epidermal growth factor, and fibroblast growth factor have no effect on the transformed cells, although epidermal growth factor can replace transforming growth factors produced by KiMSV-transformed cells for the growth of rat-1 cells. Growth of the transformed cells in serum-free medium at clonal densities is dependent upon the presence of conditioned medium collected from the same cells grown to high densities. These results show that (*i*) growth in serum-free/mitogen-free medium is a general property of KiMSV-transformed cells and (*ii*) growth of the transformed cells in this medium is dependent upon the presence of growth factors known to be produced by the cells, and they provide support for the hypothesis that serum-free growth of KiMSV-transformed cells is dependent upon ectopically produced growth factors.**

Many oncogenically transformed cells in culture produce peptide growth factors (1–11). Typically, these growth factors have been assayed by their ability to induce a round of cell division or tritiated thymidine uptake in density-inhibited or serum-restricted heterologous cells. However, a novel class of growth factors produced by cells transformed spontaneously (7, 8), chemically (11), or by Moloney murine sarcoma virus (MoMSV) (6), Kirsten murine sarcoma virus (KiMSV) (8), and simian virus 40 (SV40) (10), recently has been identified in serum-free conditioned medium from these cells. These growth factors—named transforming growth factors [TGF(s)]—have the unique ability to induce normal rat and mouse cell lines to grow when suspended in soft agar, an assay that is well correlated with tumorigenicity (12, 13). Additionally, the factors from MoMSV-transformed 3T3 cells (sarcoma growth factor) and KiMSV-transformed normal rat kidney (KNRK) fibroblasts (TGF-KNRK) cause normal cells to appear to be morphologically transformed (6, 8).

TGF-KNRK also causes normal rat and mouse cell lines to have increased rates of hexose uptake, to lose actin cable organization, to grow to high cell densities, and to grow in low serum concentrations (8). This last property of transformed cells might be explained in three ways. First, the transformed cells could be more sensitive to the same growth factors that stimulate growth of normal cells. Second, transformed cells might not require growth factors to initiate and maintain growth. Finally, the transformed cells might utilize ectopically produced growth factors in place of serum mitogens.

We sought to determine whether TGF(s) produced by KiMSV-transformed cells are involved in rendering the cells serum-independent. We report here that KiMSV-transformed rat and mouse cell lines are capable of continual growth in a defined serum-free medium devoid of exogenous mitogenic growth factors. Growth of the cells at clonal densities is dependent upon the addition of TGF(s). Normal rat-1 cells and rat-1 cells transformed by a mutant of KiMSV that is temperature sensitive for the maintenance of transformation and held at the nonpermissive temperature grow in the same serum-free medium in response to TGF(s) from the transformed cells. These results support the contention that growth factors produced by KiMSV-transformed cells can act ectopically and that these TGF(s) are intimately involved in reducing the serum requirement of the cells.

MATERIALS AND METHODS

Cells. The following cells and their sources were used in this study: normal rat kidney (NRK) and KNRK, Natalie Teich, Imperial Cancer Research Fund, London; rat-1, Claudio Basilico, New York University; KiMSV-transformed rat-1 (WT-2), Alan Horwich, ICRF; ts 371 (14)-transformed rat-1 (ts6), BALB/c 3T3, and KiMSV-transformed BALB/c 3T3 (KA31), Stuart Aaronson, National Institutes of Health. All cells were maintained in Dulbecco's modified Eagle's medium (DME medium) (GIBCO), supplemented with 10% (vol/vol) calf serum (Sterile Systems, Logan, UT) in Nunc plastic dishes (Southland Cryogenics, Dallas) in a humidified CO_2 incubator at 37°C. Subculturing was done with 0.25% trypsin/0.1 mM EDTA (GIBCO) in Ca^{2+}-, Mg^{2+}-free Dulbecco's phosphate-buffered saline.

Growth Curves. Cells were removed from dishes with trypsin, rinsed in serum-free DME medium, and then were resuspended at the indicated seeding densities in DME medium/Ham's F12 nutrient mixture (F12) (GIBCO) (1:1) supplemented with 10 mM Hepes (Sigma) at pH 7.3. Curve studies were done in 1 ml of medium in 16-mm-diameter wells (Costar, Waltham, MA) or in 2 ml of medium in 35-mm-diameter dishes (Nunc). Cells were fed every 3 days.

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Abbreviations: TGF(s), transforming growth factor(s); EGF, epidermal growth factor; KiMSV, Kirsten murine sarcoma virus; NRK, normal rat kidney; KNRK, KiMSV-transformed NRK; DME medium, Dulbecco's modified Eagle's medium; F12, Ham's F12 nutrient mixture; MoMSV, Moloney murine sarcoma virus; SV40, simian virus 40.

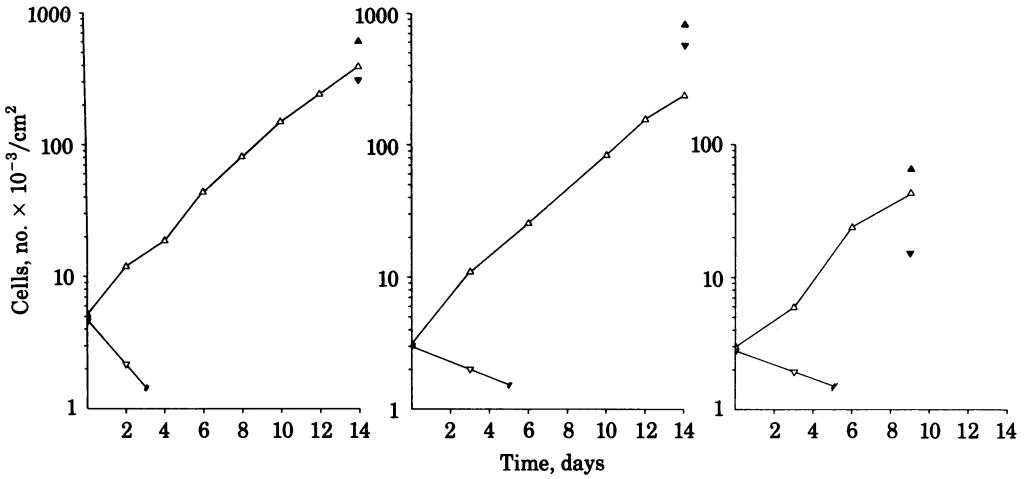

FIG. 1. Growth of KiMSV-transformed cells in serum-free/mitogen-free medium. Cells were seeded into serum-free medium in 16-mm-diameter wells at 5 × 10^3 cells per cm^2/1 ml in DME medium/F12 (1:1) supplemented with transferrin (5 μg/ml) and insulin (10 μg/ml). *Left*, NRK cells; *Middle*, Rat-1 cells; *Right*, BALB/c 3T3 cells. △, KiMSV-transformed cells; ▽, Nontransformed cells; ▲, KiMSV-transformed cells at the highest attainable density in 10% calf serum; ▼, KiMSV-transformed cells at the highest attainable density in 1% calf serum.

For the growth curves shown in Fig. 1, pictures of representative fields of cells were taken with a Polaroid camera attachment to a Leitz Diavert inverted microscope. The cells in the known area of the picture were counted. For the other growth curves the cells were removed from the dishes by addition of EDTA to a concentration of 10 mM directly to the dishes, and the cells were counted in a Coulter Counter (Coulter). Each experiment was performed at least three times and the mean values are presented. For the pictures shown in Fig. 3, a 35-mm camera attachment to the Leitz Diavert microscope was used with Kodak Tri-X Pan film (ASA 400).

Supplements used in the growth curves (stored frozen at −20°C as 100× the stock concentrations) were transferrin (Sigma), insulin (Sigma), epidermal growth factor (EGF) (Collaborative Research, Cambridge, MA), and fibroblast growth factor (Collaborative Research). The collection of serum-free conditioned medium from transformed cells and the partial purification of TGF by DEAE-cellulose chromatography was done as described (8).

RESULTS

Although many transformed cells produce factors that are tested on normal cells, the role of TGF(s) in transformation remains unclear. To show that TGF(s) known to be produced by KiMSV-transformed rat and mouse cells can act ectopically, conditions were sought in which cell growth would be dependent upon TGF(s). The serum-free growth conditions for KNRK, WT-2, and BALB/c 3T3 (KA31) cells were determined.

Fig. 1 shows that these KiMSV-transformed cells were capable of multiple rounds of division in a serum-free/mitogen-free medium consisting of DME medium/F12 (1:1) supplemented with 10 mM Hepes at pH 7.3, transferrin (5 μg/ml), and insulin (10 μg/ml). The nontransformed parent cells died rapidly in the same medium.

Growth of the transformed cells was not due to residual contamination of serum since no growth occurred in the absence of transferrin (Table 1), and each of these transformed cells was passaged for at least 3 months in this medium with no change in growth rate. Two of these lines (WT-2 and KA31) were absolutely dependent upon the presence of insulin for growth. However, KNRK cells required only transferrin (Table 1). Growth of these cells in serum-free medium was similar to growth in 1% calf serum (Table 1; Fig. 2); the latter growth was 40–60% of that achieved in 10% serum.

To determine whether addition of other known mitogens might enhance the growth of the KiMSV-transformed cells in serum-free medium, various concentrations of EGF (15) and fibroblast growth factor (16) were tested. Neither increased the growth of the transformed cells (Table 1). These preparations of growth factors were active on other cells (data not shown).

The growth of KiMSV-transformed cells in serum-free medium was dependent upon the expression of the transformed phenotype because rat-1 cells transformed by a mutant of KiMSV, which is temperature sensitive for the maintenance of transformation, only grew in serum-free medium at the permissive temperature (Table 2). At the nonpermissive temperature the cells (ts6 cells) did not grow. WT-2 cells grew at either temperature. ts6 cells were previously shown to produce thermostable (wild-type) TGF(s) only at the permissive temperature (8). When the ts6 cells at the restrictive temperature were grown in the presence of conditioned medium from the transformed cells, growth was comparable to WT-2 cells under the same conditions (Table 2). This suggests that TGF(s) can render normal cells independent of serum-derived mitogens.

When nontransformed rat-1 cells were grown in serum-free medium growth was dependent upon the addition of exogenous mitogens. Table 3 shows that TGF(s) stimulated rat-1 cells to grow as well as they did in 10% serum. EGF also stimulated

Table 1. Effect of serum-derived mitogens on the growth of transformed cells

	KNRK	WT-2	KA31
Calf serum, 10%	40.9	60.6	30.0
Calf serum, 1%	26.9	29.1	13.3
No additions	<0.25	<0.25	<0.25
T	8.4	3.3	2.0
T + I	10.7	21.7	8.8
T, I, + EGF	8.7	26.4	3.9
T, I, + FGF	8.1	26.4	3.9

Growth curves as in Fig. 2. Cells were seeded at 2 × 10^4 cells per cm^2/2 ml. The data presented are the ratios of the cell numbers at day 6 divided by the cell numbers seeded. A value of <1 indicates cell loss; 1 indicates no change in cell number; 2 indicates one cell doubling; 4 indicates two cell doublings, etc. Similar results were obtained with each growth factor at 100 ng/ml. EGF, 10 ng/ml; T, transferrin (5 μg/ml); I, insulin (10 μg/ml); FGF, fibroblast growth factor (10 ng/ml).

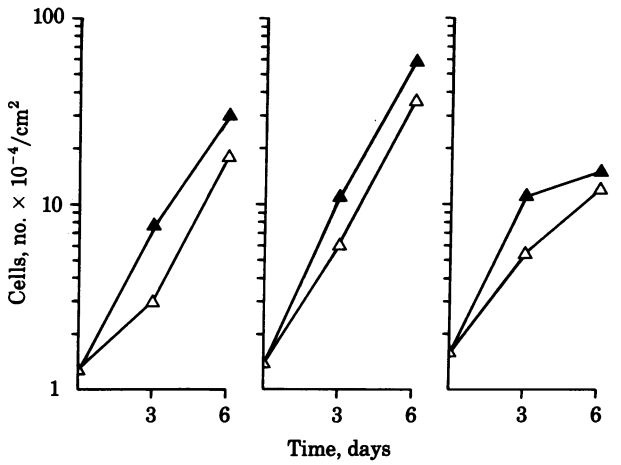

FIG. 2. Comparison of growth in serum-free/mitogen-free medium to growth in serum-supplemented medium. Cells were seeded into serum-free/mitogen-free medium supplemented with transferrin and insulin (△) or with 1% calf serum (▲) at 2 × 10^4 cells per cm^2/2 ml. *Left*, KNRK cells; *Middle*, WT-2 cells; *Right*, KA31 cells.

the growth of rat-1 cells although not to the same extent as did TGF(s). However, unlike KiMSV-transformed cells, rat-1 cells did not grow when directly seeded into serum-free medium even in the presence of TGF(s) or EGF. For cells to respond mitogenically they must be seeded in the presence of low serum concentrations. After the cells were rinsed three times with phosphate-buffered saline, their growth was dependent upon addition of either TGF(s) or EGF. This effect may be due to adhesion factors present in serum because rat-1 cells must adhere to the plate to respond to mitogens (unpublished data) or rat-1 cells may require low concentrations of a serum-derived factor no longer required by transformed cells.

From these results it appears that cells that do not produce TGF(s) are dependent upon exogenous factors for growth when placed in serum-free medium. To demonstrate that transformed cells also required mitogens for growth, it was necessary to place them in conditions such that they could not utilize the factors they produced.

Cells seeded at 2 × 10^2 cells per cm^2/2 ml (Fig. 3*A*) did not grow after 14 days even in the presence of EGF (*B*). However, cells seeded at the same density but supplemented with TGF(s) underwent extensive proliferation (*C*). When seeded at 2 × 10^3 cells per cm^2/2 ml, KNRK cells proliferated in the absence of any additives (*D*) or when supplemented with EGF (*E*), although the addition of TGF(s) again stimulated proliferation (*F*). At higher cell densities (2 × 10^4 cells per cm^2/2 ml) no additional effect of TGF(s) was observed (data not shown), presumably because enough TGF(s) were being produced for maximal growth to occur. These results establish that KNRK cells can respond to the growth factors that they produce; this constitutes an autocrine system. Similar results were observed by using WT-2 cells (data not shown).

Table 2. Growth of ts6 cells in serum-free medium

Temperature, °C	ts6/311	ts6/315	WT-2
33	15.0	14.9	5.2
39	<0.25	<0.25	26.7
39 + TGF(s)	30.0	20.8	ND

Data are presented as in Table 1 with the exception that experiments were for 11 days. Addition of TGF(s) was as a 1:4 dilution of crude conditioned medium from KNRK cells. Cells were seeded at approximately 5 × 10^3 cells per cm^2/ml and were supplemented with transferrin and insulin. ND, not done.

Table 3. Growth of rat-1 cells in serum-free medium

	Rat-1
Calf serum, 10%	22.9
Calf serum, 1%	11.8
T	<0.5
T + I	3.0
T, I, + TGF(s)	17.6
T, I, + EGF	7.0

Growth curves as in Fig. 2 with the exception that the cells were seeded in 1% calf serum. After 3 hr the cells were rinsed three times with Dulbecco's phosphate-buffered saline. Data are calculated as in Table 1. Addition of TGF(s) was as a 1:30 dilution of DEAE-cellulose partially purified TGF(s) from KNRK cells (8). Cells were seeded at 2 × 10^4 cells per cm^2/2 ml. T, I, and EGF as in Table 1.

DISCUSSION

KiMSV-transformed cells grow in serum-free/mitogen-free medium and release TGF(s) that induce normal cells to assume many characteristics of transformed cells (8). This now includes growth in serum-free medium. To determine whether the TGF(s) from KiMSV-transformed cells were responsible for the ability of KiMSV-transformed cells to grow in serum-free medium we established that KiMSV-transformed cells grew extensively in medium lacking exogenous mitogens.

The transformed cells underwent continual growth when seeded at 2 × 10^4 cells per cm^2/2 ml in a serum-free/mitogen-free medium with transferrin and—frequently but not always—insulin as the only supplements. Normal serum-derived mitogenic peptides such as EGF and fibroblast growth factor did not stimulate growth of the transformed cells in serum-free medium, demonstrating that the cells may no longer require an exogenous mitogenic signal. However, the cells were not totally independent of mitogens. When WT-2 or KNRK cells were seeded at very low cell densities (2 × 10^2 cells per cm^2/2 ml) in media without mitogens their growth was inhibited severely. This inhibition was overcome by the addition of homologous TGF(s).

Because the cells at higher cell densities did not respond to exogenous mitogens, we propose that they constitute an autocrine system that utilizes the growth factors [TGF(s)] that they produce. Why KNRK cells failed to respond to EGF at low cell density but were stimulated by TGF(s) is unclear, especially because TGF(s) contain a factor that competes with EGF for binding to cells (8). Perhaps KiMSV transformation renders the cells resistant to EGF's mitogenic signal and TGF(s) are substituted or other mitogenic factors produced by the cells are required.

The production of TGF(s) by KiMSV-transformed cells was previously shown to be controlled by the virus (8). Cells transformed by a mutant of KiMSV temperature sensitive for transformation only produced TGF(s) at the permissive temperature. Here we demonstrated that the cells transformed with the temperature-sensitive mutant of KiMSV only grew in mitogen-free medium at the permissive temperature. At the nonpermissive temperature at which ts6 cells behaved like rat-1 cells no growth was observed unless TGF(s) were added to the medium. Serum-free growth apparently is another characteristic of KiMSV-transformed cells and, like many other traits, can be mediated by TGF(s) even in the absence of the KiMSV gene product.

The enhancement of growth of KiMSV-transformed cells at low density by the autologous TGF(s) demonstrates that the cells can function as an autocrine system and provides evidence that TGF(s) could be involved in maintaining the transformed phenotype. However, the requirement of TGF(s) for the

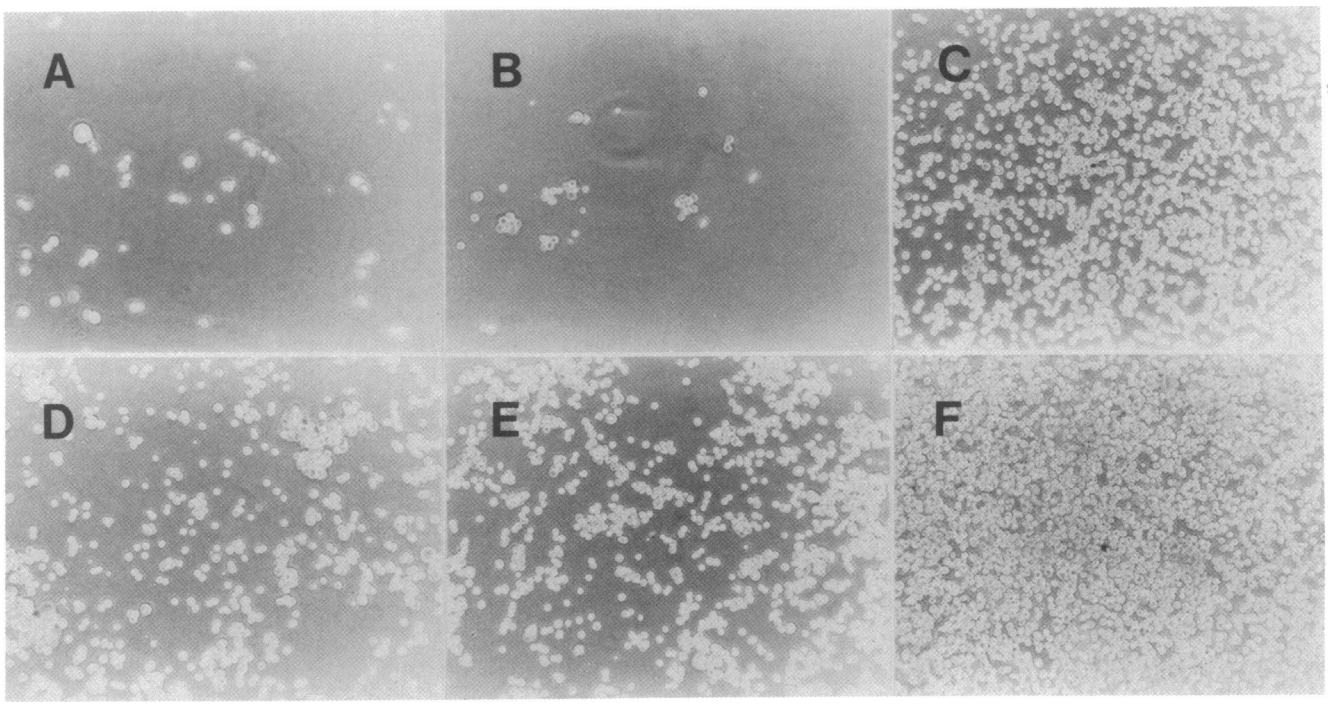

FIG. 3. Density-dependent growth of KNRK cells in serum-free medium. KNRK cells were seeded into 3.5-cm plates in serum-free medium. The cells were fed at 7 days and pictures were taken at 14 days. The cells were indistinguishable from their serum-grown counterparts. (*A–C*) Seeding density of 2×10^2 cells per cm^2/2 ml; (*D–F*) seeding density of 2×10^3 cells per cm^2/2 ml; (*A* and *D*) no additions; (*B* and *E*) EGF, 10 ng/ml; (*C* and *F*) 1:4 dilution of homologous conditioned medium (8).

expression of the fully transformed phenotype in KiMSV-transformed cells was not established by these results. Prior work with temperature-sensitive mutants of KiMSV and revertants of KiMSV transformants established a good correlation between production of TGF(s) and transformation (8). Furthermore, because TGF(s) can induce many facets of the transformed phenotype in cells that lack KiMSV genome—including growth in serum-free medium—it seems reasonable that one function of the KiMSV gene product p21 (17) is to induce TGF(s). Clearly, this does not seem to be the only function of p21 because growth of the ts6 cells was stimulated in serum-free medium at the restrictive temperature for transformation without first being seeded in serum, a requirement for the response of rat-1 cells. The cells' ability to respond to growth factors might also be altered by p21.

Growth in serum-free/mitogen-free medium was not restricted to KiMSV-transformed cells, nor was the production of TGF(s) restricted to murine sarcoma virus transformation. SV40-transformed mouse cells grow in a medium devoid of exogenous mitogens (18). SV40-transformed cells have been shown to produce growth factors (5, 10) that apparently are responsible for maintaining anchorage-independent growth (10).

Additionally, many transformed cells—including those transformed by polyoma virus, Abelson murine leukemia virus, and Rous sarcoma virus, spontaneously, and by exposure to chemicals—grow in the serum-free medium described here (unpublished data). It is not yet clear whether this indicates growth factor production in every case; however, the polyoma virus transformants and the spontaneous and chemical transformants all produce TGF(s) that can induce anchorage-independent growth of normal cell lines. A number of human tumor lines and chemically transformed mouse cell lines not yet shown to grow in serum-free medium (7, 11) have been shown to produce TGF(s).

The production of TGF(s) seems to be a widespread property of transformed cells. Here we have demonstrated for at least one transformation system (KiMSV) that production of TGF(s) enables the cells to grow in the absence of other mitogens. This indicates that the factors can act ectopically and might be responsible for other facets of the transformed cell phenotype. After all, TGF(s) induce normal cells to display many traits of transformed cells, including anchorage-independent growth which correlates well with tumorigenicity (12, 13).

Note Added in Proof. Kaighn *et al.* (19) have recently shown that the growth of two metastatic prostatic carcinoma lines, PC-3 and DU 145, in serum-free medium is density dependent and is enhanced at low densities by the addition of homologous conditioned medium.

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