IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY
Volume 24, Number 1, January 1988

GROWTH OF CELLS ON A PERFLUOROCARBON-MEDIUM INTERPHASE: A QUANTITATIVE ASSAY FOR ANCHORAGE-INDEPENDENT CELL GROWTH

BRUNELLA SANFILIPPO, FORTUNATO CIARDIELLO, DAVID S. SALOMON, AND WILLIAM R. KIDWELL[1]

Laboratory of Tumor Immunology and Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

(Accepted 13 November 1987; editor David W. Barnes)

SUMMARY

A high density, purified, nontoxic solvent, heptacosafluorotributylamine (FC43), was successfully used as a culture surface for growing several normal and oncogene-transformed cell lines under anchorage-independent conditions. Normal rat kidney (NRK) fibroblasts and the normal mammary epithelial cell lines NMuMG and A1, clone N4, of murine and human origin, respectively, failed to grow at a FC43 growth medium interphase or in soft agar in the absence of transforming growth factor alpha (TGFα) and transforming growth factor beta (TGFβ). However, NRK fibroblasts transformed with the Kirsten *ras* viral oncogene (K-NRK) or NMuMG cells transformed with a point-mutated c-Harvey-*ras* proto-oncogene or polyoma middle T-transforming gene (NMuMG-*ras*^{H} and NMuMG-*pyt*, respectively) exhibited rapid growth and formed large colonies when cultured on an FC43-medium interphase. In addition, NRK cells treated with TGFα and TGFβ and K-NRK cells grown on FC43 exhibited a sensitivity to the growth inhibitory effects of 4-*cis*-L-hydroxyproline comparable to that observed for the same cells grown in soft agar. These results demonstrated that the two-phase assay system (FC43-growth medium interphase) may be superior to soft agar for monitoring the anchorage-independent growth of cells because of the ease of cell plating, the ability to recover cells and secreted products from the upper aqueous phase, and the shorter growth period required to complete the assay (3–4 days).

Key words: anchorage-independent cell growth; two-phase system; transforming growth factor response; 4-*cis*-L-hydroxyproline sensitivity.

INTRODUCTION

There is an intimate relationship between cell growth and the composition of substrate on which cells can propagate (2,5,6–9,14,15,17,20,22). For example, normal and transformed cells exhibit different patterns of growth and different requirements for specific growth factors if they are grown on solid surfaces (glass, plastic) or in semisolid medium (soft agar, methylcellulose) (1,3,21,27). Normal fibroblasts and epithelial cells not only require a hydrophilic surface but also a rigid scaffolding in order to facilitate attachment, spreading, and growth (20). These requirements can be variably fulfilled by extracellular matrix components, such as collagen, fibronectin, and laminin (8,17). In addition, the mitogenic response to several different growth factors of certain cell types can be enhanced depending on the composition of the extracellular matrix on which the cells rest (24). Conversely, a variety of growth factors and hormones that behave as mitogens affect cellular proliferation by modulating the synthesis and/or the turnover of the extracellular matrix proteins (9,19).

The first attempt to grow cells on liquid surfaces (such as siliconated and fluorinated hydrocarbons) was made by Rosenberg in 1964 (21). He found that normal conjunctival cells were able to spread and grow on

[1] To whom correspondence should be addressed at Laboratory of Tumor Immunology and Biology, NCI, NIH, Building 10, Room 5B39, Bethesda, MD 20892.

fluorocarbon fluids containing impurities. However, if a layer of lecithin was injected between the culture medium and the fluorocarbon phases, spreading of the cells was markedly reduced. Cells apparently did not directly interact with the fluorocarbon fluid surface but rather with a layer of denaturated serum proteins that were present in the culture medium. The active impurity found in fluorocarbon fluids was proposed to be an amphipathic molecule that could bind to serum proteins and thus promote the formation of a rigid surface on which cells could spread and grow as a monolayer.

Purified fluorocarbon fluids were found to be poor substrates for normal anchorage-dependent cell growth because the normal cells could not spread on the fluorocarbon surface. We now show that normal cells can grow on pure FC43, provided transforming growth factors are added.

Normal rat kidney (NRK) cells have been routinely utilized to screen for growth factors that induce anchorage-independent growth in soft agar (1,3,4,21). These cells were therefore selected to ascertain if growth factors such as transforming growth factor α (TGFα) and β (TGFβ) could facilitate the growth of NRK cells on a fluorocarbon-medium interphase (two-phase assay). In addition, a comparison was also made between the growth of TGF-treated nontransformed cells, such as NRK cells, and two mammary epithelial cell lines and their oncogene-transformed counterparts on a fluorocarbon-medium interphase. Growth of NRK cells in soft agar requires the presence of both TGFα and TGFβ (1,21). Moreover, TGFα is capable of stimulating the anchorage-independent growth of mammary epithelial cells (25). The anchorage-independent growth of a number of transformed rodent cell lines and of TGF-treated NRK cells can be inhibited by 4-cis-L-hydroxyproline (CHP). CHP is an analog of proline that can selectively block the synthesis and secretion of collagen when used at low concentrations (23,28) and can induce the regression of primary carcinogen-induced rat mammary adenocarcinomas through this mechanism (16,18).

The present study demonstrates that transformed NRK cells and transformed mouse mammary epithelial cells are able to actively grow as colonies on a heptacosafluorotributylamine (FC43)-medium interphase even in the absence of TGFs, while NRK cells or mammary epithelial cells exhibit a comparable set of requirements for TGFα and for TGFβ for growth in soft agar and in the two-phase assay system. In addition, TGF-treated NRK or Ki-*ras*-transformed NRK cells, K-NRK, have an equivalent degree of sensitivity to the growth inhibitory effects of CHP in the two-phase assay system and in soft agar.

Materials and Methods

Cell culture. NRK fibroblasts, clone F49, were obtained from Dr. J. E. DeLarco, National Cancer Institute, FCRC, Ft. Detrick (Frederick, MD). Kirsten murine sarcoma virus-transformed NRK cells (K-NRK) were kindly provided by Dr. R. E. Bassin, National Cancer Institute (Bethesda, MD). Normal murine mammary epithelial cells (NMuMG) and their transformed counterparts with an activated c-Harvey-*ras* and polyoma middle T oncogene, NmuMG-*ras*^{H} and NMuMG-*pyt*, respectively, were obtained from Dr. N. Hynes, the Ludwig Institute for Cancer Research (Bern, Switzerland). All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), glutamine (4 m*M*), penicillin (100 U/ml), streptomycin (100 μg/ml), and Hepes (20 m*M*). The normal human mammary epithelial cell line A1, clone N4, was originally derived from a reductive mammoplasty. The cells were immortalized by benzo-α-pyrene treatment of the primary human mammary epithelial cell cultures. The A1 cells were obtained from Dr. M. Stampfer, Lawrence Berkeley Laboratory (Berkeley, CA). They were grown in DMEM-F12, 1:1 mixture, supplemented with 0.5% FBS, insulin (10 μg/ml), transferrin (10 μg/ml), hydrocortisone (0.5 μg/ml) epidermal growth factor (EGF), culture grade (10 ng/ml; Collaborative Research, Lexington, MA), penicillin (100 U/ml), and streptomycin (100 μg/ml). The transformed cell lines (K-NRK, NMuMG-*ras*^{H}, and NMuMG-*pyt*) were capable of growing in soft agar and of

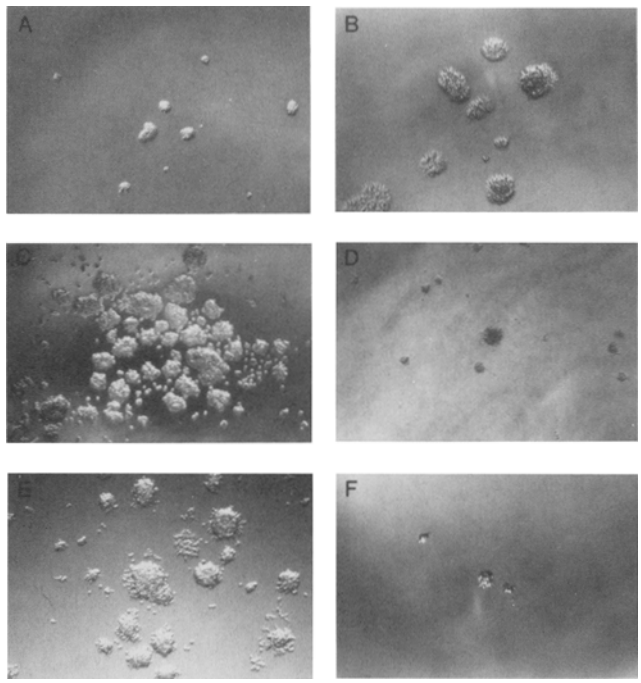

FIG. 1. Effect of TGFs on NRK cell growth and CHP sensitivity of K-NRK cells in two-phase assay system. NRK cells were grown on a fluorocarbon FC43 surface for 4 days, as described in "Materials and Methods," in the absense of TGFs (*A*) or in the presence of TGFα (2 ng/ml) (*B*), TGFα (2 ng/ml) and TGFβ (1 ng/ml) (*C*), or TGFβ (1 ng/ml) (*D*). K-NRK cells were grown for 4 d on FC43 in the absence of TGFs (*E*) or were treated with CHP (250 μg/ml) (*F*). ×250.

producing tumors when injected into nude mice. Neither of these properties was associated with the normal cell lines NRK, NMuMG, and A1N4 (25,26).

Fluorocarbon purification. Heptacosafluorotributylamine (FC43) was purchased from Sigma Chemical Co. (St. Louis, MO) and purified through columns of alumina (neutral Brockman activity I, 80–200 mesh, Fisher) as described by Keese and Giaever (14) to remove the amphipathic contaminant that facilitates cell spreading. Before use, FC43 was filtered through a 0.2-μm Nalgene filter.

Preparation of two-phase system for anchorage-independent growth assay. After purification and filtration, 0.5 ml of FC 43 was added to cylindrical wells of a 48-well tissue culture cluster dish (Costar, Cambridge, MA). The same volume of cell suspension in culture medium (2.5×10^3 cells/ml) was gently pipetted on the top of the fluorocarbon phase to form a second phase. Because of the different densities and the different chemical characteristics, the two liquid phases do not mix, which allows the cells to grow at the fluorocarbon-medium interface under anchorage-independent conditions. In the two-phase assay system, the cells were cultured for 4 days at 37° C in a humidified atmosphere of 5% CO_2. Cell growth was monitored by determining the incorporation of labeled [^{14}C]thymidine (50–60 mCi/mmol, 1 μCi/ml; Amersham, Arlington Heights, IL) into DNA after labeling of the cells for the last 48 h of culture.

Recovery of cells from the two-phase assay system and evaluation of [^{14}C]thymidine incorporation. After 4 days of culture, the [^{14}C]thymidine-labeled cells were recovered from the 48-multiwell tissue culture dishes by pipetting the aqueous culture medium phase onto 2.4 cm glass fiber filters (Whatman GF/C, Maidstone, England). The cells were trapped by the filters, and the filters were subsequently washed several times with phosphate-buffered saline (PBS) and 5% ice-cold trichloracetic acid (TCA) containing 0.05% unlabeled thymidine (Calbiochem, San Diego, CA) and finally with absolute methanol. After 10 min at 80° C in a vacuum oven, the filters were counted in a toluene PPO-based counting solution.

Response of cells to TGFs and CHP in the two-phase system assay. Normal cells (NRK, NMuMG, and A1N4) were grown in the presence or absence of TGFα (human recombinant TGFα, 2 ng/ml), generously supplied by Dr. Ryk Derynck, Department of Molecular Biology, Genentech, Inc. (San Francisco, CA), and/or TGFβ1 from porcine platelets (1–2 ng/ml; R & D System, Inc., Minneapolis, MN), while transformed cells (K-NRK, NMuMG-*ras*^{H}, and NMuMG-*pyt*) were grown without TGFs. The sensitivity of NRK cells grown in the absence or presence of TGFα and TGFβ and of K-NRK cells to different concentrations of CHP (5–100 μg/ml) was tested in the two-phase assay system, in soft agar, and in monolayer cultures.

Soft agar assay. Cells (2×10^4 per well) were plated in 1 ml of 0.3 Difco nobel agar that was supplemented with DMEM and 10% FBS. Cell suspensions were layered over 1 ml of a 0.66% agar medium base layer in 12-well cluster dishes (Costar). Anchorage-independent growth in soft agar of all the cell lines used for the two-phase assay system was tested in the presence or in the absence of TGFα (2 ng/ml) and/or TGFβ (1–2 ng/ml). The sensitivity of growth factor-treated NRK cells and K-NRK cells to several concentrations of CHP (5–100 μg/ml) was also assessed. The cells were maintained in culture, and every 3 days they were fed with 0.1 ml of culture medium; after 14 days they were stained with nitro blue tetrazolium (NBT; Sigma Chemical Co.) and quantitated on an Artek Model 880 colony counter. Colonies greater than 50 μm in diameter were scored and counted.

Monolayer culture. NRK cells were seeded at a density of 15×10^4 cells/dish in 60-mm tissue culture dishes (Falcon, Lincoln Park, NJ). Several concentrations of CHP (5–100 μg/ml) were tested in the presence or in the absence of TGFα (2 ng/ml) and/or TGFβ (1 ng/ml). After 4 days of culture, the cells were trypsinized and counted in a Coulter counter (Coulter Electronics Inc., Hialeah, FL). A similar procedure was used for K-NRK cells, with the exception that no growth factors were added to the cultures.

RESULTS

Growth and morphology of cells cultured on FC43 fluorocarbon surfaces. To ascertain whether there were

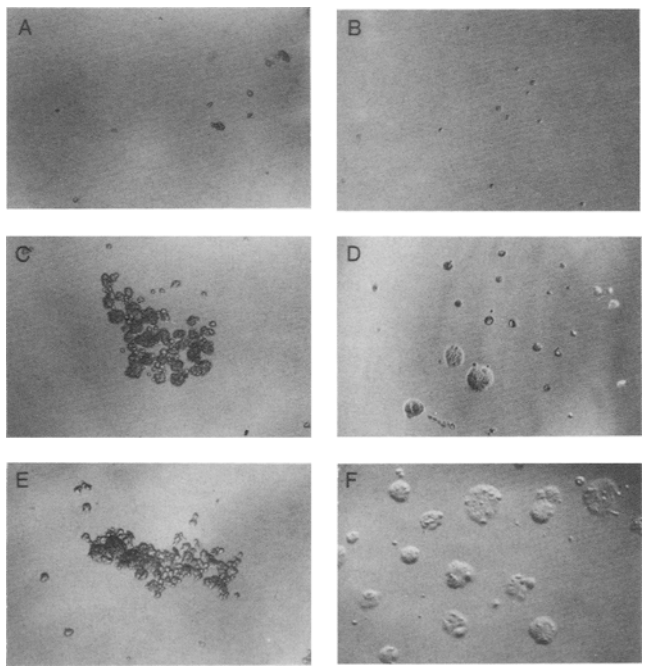

FIG. 2. NMuMG-*ras*^{H}, NMuMG, and A1N4 cell growth on FC43 in the absence or presence of TGFs. NMuMG cells were grown in the absence (*A*) or in the presence (*C*) of TGFα (2 ng/ml); NMuMG-*ras*^{H} cells were grown in the absence of TGFs (*E*); A1N4 cells were cultured in the absence (*B*) or in the presence (*F*) of TGFα (2 ng/ml) and TGFβ (1 ng/ml) and in the presence of TGFα (2 ng/ml) alone (*D*). ×250.

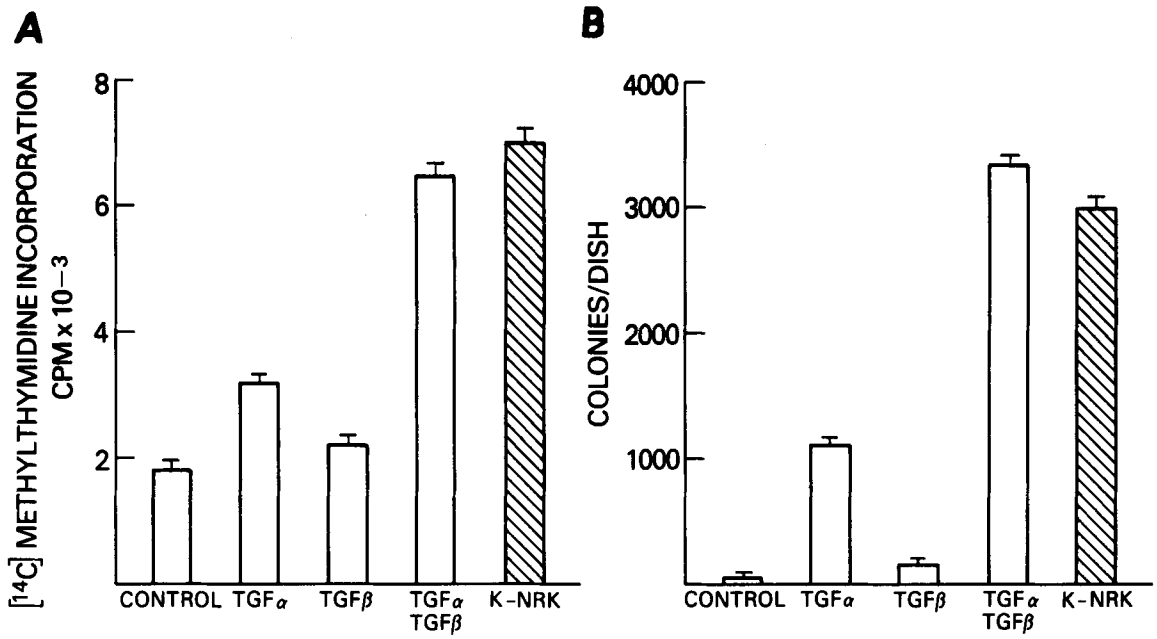

FIG. 3. [^{14}C]methylthymidine incorporation into DNA and colony formation of NRK and K-NRK cells in two-phase assay system (*A*) and in soft agar cultures (*B*). *Open bars* represent NRK cell growth in the absence (control) or presence of TGFα (2 ng/ml) and/or TGFβ (1 ng/ml). *Dashed bars* represent the growth of K-NRK cells in the absence of TGFs.

intrinsic differences in the ability of normal and transformed fibroblasts and epithelial cells to grow on a fluorocarbon-medium interphase, NRK cells and mouse and human mammary epithelial cells and their *ras*-transformed counterparts were cultured on FC43 for 4 days. As illustrated in Fig. 1*A*, nontransformed NRK cells seeded on a FC43-medium interphase in the two-phase assay system failed to form large colonies. However, when 2 ng/ml of TGFα was added to the cultures, NRK cells were able to grow as distinct large colonies (Fig. 1*B*). This effect was markedly enhanced if 1 ng/ml of TGFβ was simultaneously present in the medium (Fig. 1*C*). TGFβ alone did not stimulate the growth of NRK cells as colonies on the FC43-medium interphase (Fig. 1*D*). In constrast to NRK cells, K-NRK cells grew as colonies on FC43 in the absence of added TGFs and exhibited a morphology similar to NRK cells grown in the presence of TGFα and TGFβ (Fig. 1*E*). K-NRK cell growth

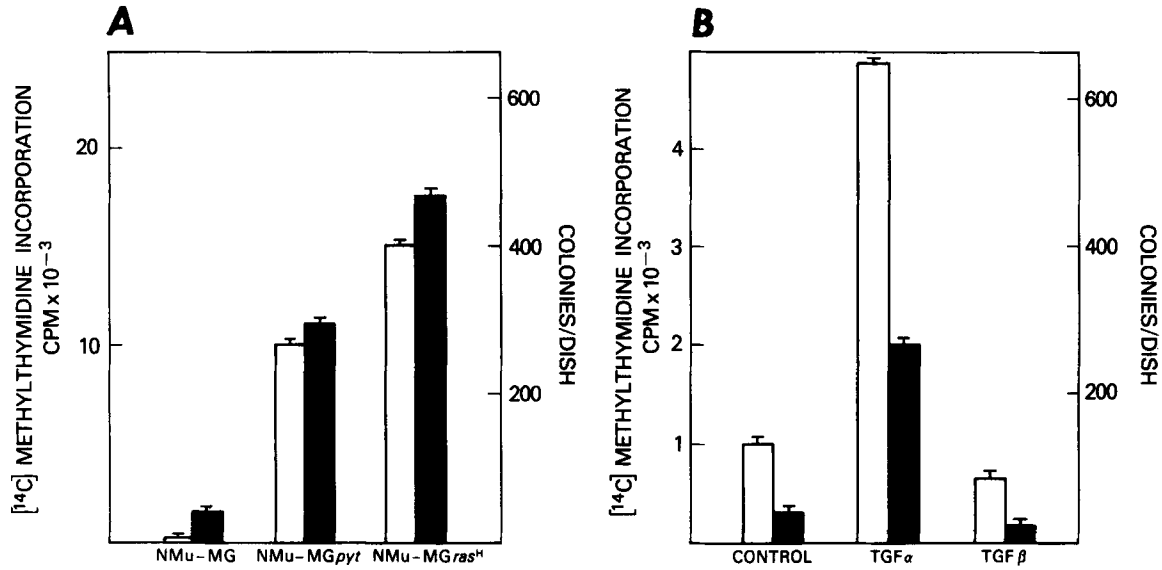

FIG. 4. (*A*) NMuMG, NMuMG-*pyt*, and NMuMG-*ras*^{H} cell growth on FC43 (*open bars*) and in soft agar (*closed bars*) in the absence of TGFs. (*B*) NMuMG cell growth in two-phase system (*open bars*) and in soft agar (*closed bars*) in the absence (control) or in the presence of TGFα (2 ng/ml) or TGFβ (1 ng/ml).

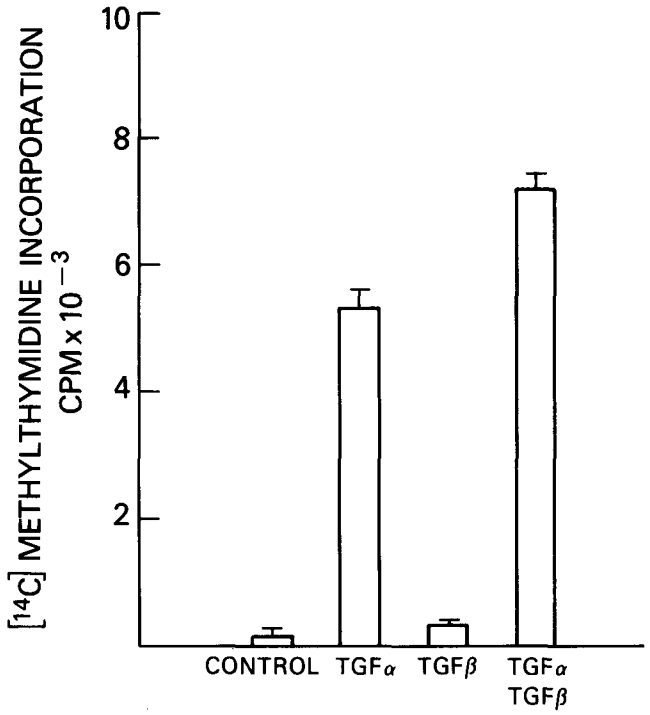

FIG. 5. A1N4 cell growth on FC43 in the absence (control) or in the presence of TGFα (2 ng/ml) and/or TGFβ (2 ng/ml).

as colonies was completely inhibited when 25 μg/ml of CHP was added to the cultures (Fig. 1*F*).

The ability of other types of cells to grow on the fluorocarbon-medium interface was then tested using mouse and human mammary epithelial cells. Fig. 2 demonstrates that, in the absence of exogenous growth factors, normal mouse mammary NMuMG epithelial cells (Fig. 2*A*) and benzo-a-pyrene immortalized human mammary epithelial A1N4 cells (Fig. 2*B*) did not grow as colonies on FC43. When 2 ng/ml of TGFα was added to the cultures, NMuMG cells and A1N4 cells (Fig. 2, *C* and *D*) grew as colonies. Addition of TGFβ alone had no effect on promoting the growth of either NMuMG or A1N4 cells (data not shown). However, TGFβ (1 ng/ml) was able to synergistically potentiate the growth promoting effects of TGFα on the A1N4 cells (Fig. 2*F*) but not on the growth of NMuMG cells (data not shown). NMuMG cells transformed with an activated c-Ha-*ras* proto-oncogene, NMuMG-^{H}, were able to grow as colonies on the FC43 fluoro-carbon interface in the absence of exogenous growth factors and exhibited a morphology similar to TGFα-treated NMuMG cells (Fig. 2*E*).

Quantitation of growth factor response of cells growing in soft agar and on FC43. The mitogenic response of these various cell lines to TGFα and/or TGFβ was quantitated by monitoring the incorporation of [^{14}C]methylthymidine into DNA for growth on the FC43 fluorcarbon interface and was compared with the growth of these cells in soft agar as colonies. The colony formation ability for all the cell lines on FC43, as well as in the soft agar cultures, was quantified by staining with NBT and counting the colonies on the colony counter (data not shown). Fig. 3*A* demonstrates that NRK cell growth on FC43 increased approximately 70% after the addition of TGFα (2 ng/ml). Addition of TGFβ alone had no effect on growth but acted synergistically with TGFα to enhance NRK cell growth (approximately 3-fold and comparable to the level observed with K-NRK cells in the absence of growth factors). Fig. 3*B* demonstrates that qualitatively similar results were observed for the effects of TGFα and β on NRK and K-NRK cells grown in soft agar. Normal NRK cells did not grow in the absence of TGFα and TGFβ or in the presence of TGFβ alone. TGFα alone stimulated the anchorage-independent growth of NRK cells somewhat, whereas the addition of TGFα and TGFβ induced colony formation of NRK cells to a level comparable to that observed for K-NRK cells.

The growth of normal and oncogene-transformed mouse mammary epithelial cells on FC43 and in soft agar was then compared (Fig. 4*A*). While normal NMuMG cells did not form colonies either in soft agar or on FC43, NMuMG-*pyt* and NMuMG-*ras*^{H} cells grew as colonies in both conditions. In addition, the degree of growth for both the NMuMG-*pyt* and NMuMG-*ras*^{H} cells was similar on FC43 and in soft agar. A similar cellular response in the two assays was observed when the growth of NMuMG cells was assessed in the absence or in the presence of TGFα or TGFβ (Fig. 4*B*). Addition of TGFα (2 ng/ml) induced a 5-fold increase in labeled thymidine incorporation into DNA in NMuMG cells on FC43 and in colony formation in soft agar. TGFβ (1 ng/ml) alone failed to stimulate NMuMG cell growth in either assay and was, in fact, slightly inhibitory in both assays. Fig. 5 illustrates a similar growth response of the A1N4 cells to

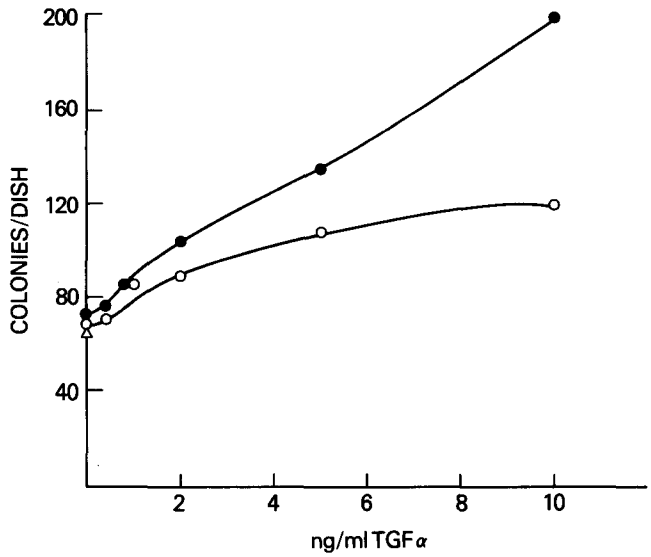

FIG. 6. Growth of A1N4 cells in soft agar. A1N4 cells were treated with different concentrations of TGFα (2-10 ng/ml) in the absence (○) or in the presence (●) of TGFβ (2 ng/ml); A1N4 cell growth in the presence of TGFβ only (△).

TGFs. Addition of TGFα (2 ng/ml) induced a 12-fold increase in the growth of the A1N4 cells, whereas TGFβ (1 ng/ml) alone had no effect. Treatment of A1N4 cells with both TGFα and TGFβ increased the growth of A1N4 cells over that seen with TGFα alone. Fig. 6 illustrates the growth of A1N4 cells in soft agar as colonies in response to varying concentrations of TGFα in the absence or in the presence of TGFβ (2 ng/ml). TGFα (5–10 ng/ml) produced a 2-fold increase in A1N4 colony formation. Addition of TGFβ alone had no effect on A1N4 growth in soft agar, whereas TGFβ synergistically enhanced the anchorage-independent growth of A1N4 cells in response to different concentrations of TGFα. Under these conditions, there was approximately a 3-fold increase in the number of colonies in the presence of TGFα (10 ng/ml) and TGFβ (2 ng/ml).

CHP-induced growth inhibition of NRK and K-NRK cells growing on FC43, in soft agar, and in monolayer cultures. As we recently found (F. Ciardiello et al., manuscript submitted), CHP-induced growth inhibition is more pronounced in a variety of transformed rodent cells and in cells, such as NRK cells, that have been grown in the presence of TGFα and TGFβ. We therefore compared the sensitivity of growth factor-treated NRK and of K-NRK cells to different concentrations of CHP when cells were cultured on FC43, in soft agar, and in monolayer cultures (Fig. 7). Fig. 7*A* demonstrates that NRK cells cultured in the presence of TGFα (2 ng/ml) and TGFβ (1 ng/ml) exhibit a similar degree of growth inhibition induced by different concentrations of CHP on FC43 and in soft agar. A 50% inhibition of NRK cell growth in both assays is observed at a CHP concentration between 5 and 10 μg/ml. K-NRK cells grown on FC43 and in soft agar also exhibited a comparable degree of sensitivity to the growth inhibitory effect of CHP and identical values as obtained for TGFα- and TGFβ- treated NRK cells. It was also observed that NRK cells grown in monolayer culture are relatively insensitive to the growth inhibitory effects of CHP (Fig. 7*B*). However, when these cells grown on monolayers were treated with TGFα and TGFβ, the cells exhibited a higher degree of sensitivity to CHP than untreated NRK cells. Inhibition was comparable to that observed with K-NRK cells under the same conditions. However, the CHP sensitivity of TGFα- and TGFβ-treated NRK cells and K-NRK cells was lower in cells grown as monolayer cultures than for the same cells grown in soft agar or on FC43 (ID_{50}, approximately 50 μg/ml).

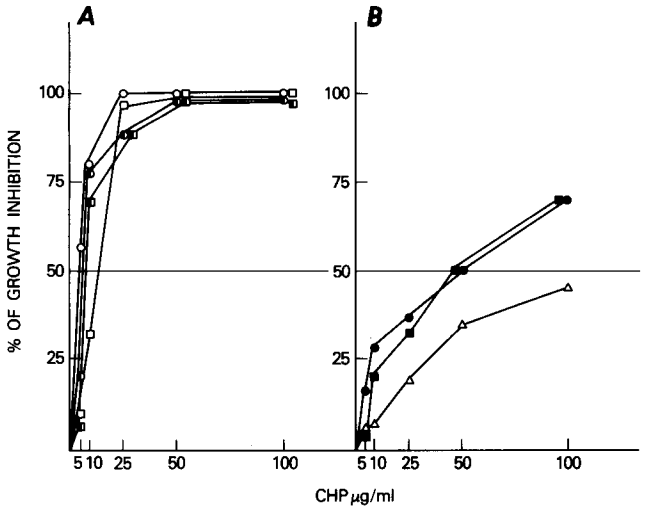

FIG. 7. CHP sensitivity of NRK and K-NRK cells grown in soft agar, in the two-phase system assay, and in monolayer cultures. (*A*) NRK and K-NRK cells were cultured on FC43 (◑, ◨) and in soft agar (○, □, respectively, and their sensitivity to several different concentrations (5–100 μg/ml) of CHP was tested. NRK cells in the two-phase system and in soft agar were grown in the presence of TGFα (2 ng/ml) and TGFβ (1 ng/ml). (*B*) NRK cell sensitivity to CHP in monolayer cultures in the presence (●) or in the absence (△) of TGFs. K-NRK cell sensitivity to CHP in monolayer cultures in the absence of TGFs (■).

DISCUSSION

Fluorocarbon fluids are a family of nontoxic compounds that exhibit a high solubility for gases such as oxygen and carbon dioxide (11,12). For these reasons, fluorocarbon emulsions have been utilized as a blood substitute (10), as microcarriers for mass culture of cells (15), and for maintaining the viability of organs for transplantation studies (13). The present experiments were designed to formulate an improved assay for rapidly measuring the anchorage-independent growth of transformed cells and of normal cells treated with TGFs, an assay that would be superior to the more conventional and time-consuming soft agar or methylcellulose assays. Our results demonstrate that purified FC43 represents a suitable growth surface on which normal cells cannot attach and spread significantly in the absence of TGFs.

The growth of nontransformed NRK fibroblasts and mouse and human mammary epithelial cells in the two-phase FC43 assay is shown to require the presence of TGFα and, in some instances (e.g., NRK cells), TGFβ. In fact, the magnitude of growth in the two-phase assay system in response to these two growth factors for NRK cells and for the mammary epithelial cells is comparable to the degree of growth stimulation produced by these two growth factors in soft agar. Furthermore, the growth of TGFα- and TGFβ-treated NRK and NMuMG mouse mammary epithelial cells is comparable in both assays to the amount of growth observed in *ras*-transformed NRK cells and *ras*-transformed NMuMG mouse mammary epithelial cells. These *ras*-transformed cell lines and the polyoma-transformed NMuMG cells exhibit equivalent growth in the absence of added growth factors in the two-phase assay system and in soft agar, supporting the observation that these two assays are approximately equivalent in their ability to monitor growth factor

stimulation of normal cell to grow as transformed cells. This observation is further supported, since the magnitude of growth inhibition produced by CHP at any given concentration in TGFα- and TGFβ-treated NRK cells and in K-NRK cells is equivalent in the two-phase assay system and in soft agar.

The two-phase system offers several major advantages over the soft agar assay normally used for monitoring the anchorage-independent growth of cells. First, the recovery of cells and secreted cellular products from the two-phase assay system is relatively simple, since it only requires aspiration to harvest the conditioned medium and filtration to collect the cells. In contrast, recovery of cell colonies from soft agar is extremely tedious and requires melting and dilution of the agar while recovery of secreted cell products requires separation of the desired product from agar, a major problem. Second, the ease of cell plating is also a major advantage of the two-phase assay. Cells do not have to be resuspended in a semisolid medium since they are grown directly on a fluorocarbon-liquid interphase. Finally, the assay can be completed within 3-4 days compared with 10-14 days for routine soft agar assays. The rapidity of this assay over conventional soft agar or methylcellulose assays should also facilitate the routine screening of other growth factors that can promote the clonal or anchorage-independent growth of different indicator cells of mesenchymal or epithelial origin.

REFERENCES

1. Anzano, M. A.; Roberts, A. B.; Meyers, C. A.; Komoriya, A.; Lamb, L. C.; Smith, J. M.; Sporn, M. B. Synergistic interaction to two classes of transforming growth factors from murine sarcoma cells. Cancer Res. 42:4776-4778; 1982.
2. Benecke, B. J.; Ben-Ze'ev, A.; Penman, S. The control of mRNA production, translation, and turnover in suspended and reattached anchorage-dependent fibroblasts. Cell 14:931-939; 1978.
3. DeLarco, J. E.; Todaro, G. J. Growth factors from murine sarcoma virus-transformed cells. Proc. Natl. Acad. Sci. USA 75:4001-4005; 1978.
4. Derynck, R.; Roberts, A. B.; Winkler, M. E.; Chen, E. Y.; Goeddel, D. V. Human transforming growth factor-α: precursor structure and expression in E. coli. Cell 38:287-297; 1984.
5. Farmer, S. R.; Ben-Ze'ev, A.; Benecke, B. J.; Penman, S. Altered translatability of messenger RNA from suspended anchorage-dependent fibroblasts: reversal upon cell attachment to a surface. Cell 15:627-637; 1978.
6. Folkman, J.; Tucker, R. W. Cell configuration, substratum and growth control. Subtelny, S.; Wessels, N. K. eds. The cell surface: mediator of developmental processes. New York: Academic Press; 1980: 259-275.
7. Giaever, I.; Keese, C. R. Behavior of cells at fluid interfaces. Proc. Natl. Acad. Sci. USA 80:219-222; 1983.
8. Gospodarowicz, D.; Greenburg, G.; Birdwell, C. R. Determination of cellular shape by the extracellular matrix and its correlation with the control of cellular growth. Cancer Res. 38:4155-4171; 1978.
9. Gospodarowicz, D.; Tuber, J.-P. Growth factors and the extracellular matrix. Endocrine Rev. 1:201-227; 1980.
10. Gould, S. A.; Rosen, A. L.; Sehgal, L. R.; Sehgal, H. L.; Langdale, L. A.; Krause, L. M.; Rice, C. L.; Chamberlin, W. H.; Moss, G. S. Fluosol-DA as a red-cell substitute in acute anemia. N. Engl. J. Med. 314:1653-1656; 1986.
11. Grand Island Biological Company. Potential uses of perfluorochemical artificial blood for experimental studies in physiology, biology, biochemistry, chemotherapy, toxicology, metabolism, etc. Fluosol-43. Technical Information Ser. No. 3, Sept. 4, 1976, RN/Ky/yd. Osaka, Japan: The Green Cross Corporation; 1976.
12. Iwai, M.; Yamada, N.; Kondo, N.; Okamoto, H.; Yokoyama, K.; Suyama, T.; Naito, R.; Ohyanagi, H.; MItsuno, T. Oxypherol. Perfusate for kidney preservation. Proceedings of the IVth International Symposium on Perfluorochemical Blood Substitutes, Kyoto; 1978: 225-236.
13. Kamada, N.; Calne, R. Y.; Wight, D. G. D.; Lines, J. G. Orthotopic rat liver transplantation after long-term preservation by continuous perfusion with fluorocarbon emulsion. Transplantation 30:43-48; 1980.
14. Keese, C. R.; Giaever, I. Cell growth on liquid interfaces: role of surface active compounds. Proc. Natl. Acad. Sci. USA 80:5622-5626; 1983.
15. Keese, C. R.; Giaever, I. Cell growth on liquid microcarriers. Science 219:1448-1449; 1983.
16. Kidwell, W. R.; Taylor, S.; Bano, M.; Grantham, F. Growth arrest of mammary tumors by proline analogs. Brescianai, F., et al. eds. Progress in cancer research and therapy. Vol. 31. New York: Raven Press; 1984: 129-135.
17. Kleinman, H. K.; Klebe, R. J.; Martin, G. R. Role of collagenous matrices in the adhesion and growth of cells. J. Cell Biol. 88:473-485; 1981.
18. Lewko, W. M.; Liotta, L. A.; Wicha, M. S.; Vonderhaar, B. K.; Kidwell, W. R. Sensitivity of N-nitrosomethylurea-induced rat mammary tumors to cis-hydroxyproline, an inhibitor of collagen production. Cancer Res. 41:2855-2862; 1981.
19. Liotta, L. A.; Wicha, M. S.; Foidart, J. M.; Rennard, S. I.; Garbisa, S.; Kidwell, W. R. Hormonal requirements for basement membrane collagen deposition by cultured rat mammary epithelium. Lab. Invest. 41:511-517; 1979.
20. Maroudas, N. G. Chemical and mechanical requirements for fibroblast adhesion. Nature 244:353-354; 1973.
21. Roberts. A. B.; Frolik, C. A.; Anzano, M. A.; Sporn, M. B. Transforming growth factors from neoplastic and nonneoplastic tissues. Fed. Proc. 42:2621-2626; 1983.
22. Rosenberg, M. D. Cell surface interactions and interfacial dynamics. Emmelot, P.; Muhlbock, O., eds. Cellular control mechanisms and cancer. Amsterdam: Elsevier Publishing Company; 1964: 146-164.
23. Rosenbloom, J.; Prockop, D. J. Incorporation of cis-hydroxyproline into procollagen and collagen. J. Biol. Chem. 246:1549-1555; 1971.
24. Salomon, D. S.; Liotta, L. A.; Kidwell, W. R. Different response to growth factors of mammary epithelium plated on different collagen substrata in serum-free medium. Proc. Natl. Acad. Sci. USA 78:382-386; 1981.
25. Salomon, D. S.; Perroteau, I.; Kidwell, W. R.; Tam, J.; Derynck, R. Loss of growth responsiveness to epidermal growth factor and enhanced production of alpha-transforming

growth factors in ras-transformed mouse mammary epithelial cells. J. Cell. Physiol. 130:397-409; 1987.

26. Stampfer, M. R.; Bartley, J. C. Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo[a]pyrene. Proc. Natl. Acad. Sci. USA 82:2394-2398; 1985.
27. Stoker, M.; O'Neill, C. Anchorage and growth regulation in normal and virus-transformed cells. Int. J. Cancer 3:683-693; 1968.
28. Wicha, M. S.; Liotta, L. A.; Vonderhaar, B. K.; Kidwell, W. R. Effects of inhibition of basement membrane collagen deposition on rat mammary gland development. Dev. Biol. 80:253-266; 1980.

EDITOR'S STATEMENT

This report describes the application of a novel technique that provides an alternative approach to assay of anchorage independence with some unique advantages over conventional methods.