IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY
Volume 21, Number 1, January 1985

ANCHORAGE-INDEPENDENT GROWTH OF NORMAL HUMAN MESOTHELIAL CELLS: A SENSITIVE BIOASSAY FOR EGF WHICH DISCLOSES THE ABSENCE OF THIS FACTOR IN FETAL CALF SERUM

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(Received 14 December 1984; editor A. B. Pardee)

SUMMARY

This laboratory recently reported that normal human mesothelial cells require epidermal growth factor (EGF) and hydrocortisone (HC), in addition to fetal calf serum and a complex defined medium component, in order to grow optimally in surface culture (9). We report here that this normal cell type also forms large colonies at high efficiency in semi-solid medium, but exhibits more stringent serum and EGF requirements for anchorage-independent than for surface growth. Mesothelial cells are unable to divide at all in semi-solid medium without added EGF or with less than 2% serum, whereas they grow slowly but progressively in surface culture under such conditions. In semi-solid medium containing 20% serum and HC, mesothelial cells are stimulated to divide by the addition of as little as 30 pg/ml purified EGF. Human urine or male mouse plasma could substitute for purified EGF, yielding growth commensurate with the levels of EGF in these biological fluids previously measured by others using radioreceptor and radioimmune assays. Thus growth of mesothelial cells in semi-solid medium can serve as a highly sensitive assay of EGF biological activity which is unaffected by the presence of serum proteins. In addition, our results demonstrate that fetal calf serum does not provide mitogenic levels of EGF to cultured cells, raising the question of the identity of plasma and serum mitogens.

Key words: EGF; mesothelial cells; anchorage-independent growth; human diploid.

INTRODUCTION

For cell types other than those of the hematopoietic system, anchorage-independent growth in culture was initially thought to be an acquired character of malignantly transformed cells (20,29). Several normal diploid cell types, however, have been found to grow well in semi-solid medium (3,19,23,33). Normal human fibroblasts, for example, form large colonies at high efficiency in semi-solid medium if high serum concentrations and hydrocortisone are provided (23). A specific serum factor for anchorage-independent growth has been isolated (24) and characterized as a platelet-derived polypeptide, "TGF-β" (8), distinct from platelet-derived growth factor (PDGF). TGF-β is also secreted by some transformed cell lines (11,21,28). EGF works synergistically with TGF-β to stimulate anchorage-independent growth of non-transformed fibroblasts, and an EGF-like polypeptide, TGF-α, has also been purified from the conditioned medium of some transformed cell lines (1).

While searching for a specific transformation phenotype that might serve to identify and permit selection of malignant mesothelial cells (mesothelioma) in culture, we found that normal human mesothelial cells grow well in semi-solid medium. We therefore sought to determine whether their EGF, HC, and serum requirements are different for anchorage-independent and anchored growth.

MATERIALS AND METHODS

Cells and culture medium. LP-9 cells are normal human peritoneal mesothelial cells, cultured from an adult female. They are diploid and have a finite replicative lifespan of about 50 population doublings (9,32). Early passage LP-9 cells have been submitted to the Aging Cell Repository of the Institute for Medical Research (Camden, NJ), where they have been tested and found free of mycoplasma, and are available from this collection as Repository #AG 7086. The anchorage-independent growth requirements were confirmed with another normal peritoneal strain, LP-3, and with a normal pleural mesothelial strain, HPM-2. Mesothelial cells were serially cultivated in plastic culture dishes with a 1:1 mixture of M199 (GIBCO) and MCDB202 (KC Biologicals, Genexa, KS) supplemented with 15-20% fetal calf serum (Sterile Systems, Logan, UT), 0.4 μg/ml hydrocortisone (Calbiochem, San Diego, CA) and 10 ng/ml mouse epidermal growth factor (Collaborative Research, Lexington, MA) (9). In order to assess anchorage-independent growth, medium was made semi-solid by the addi-

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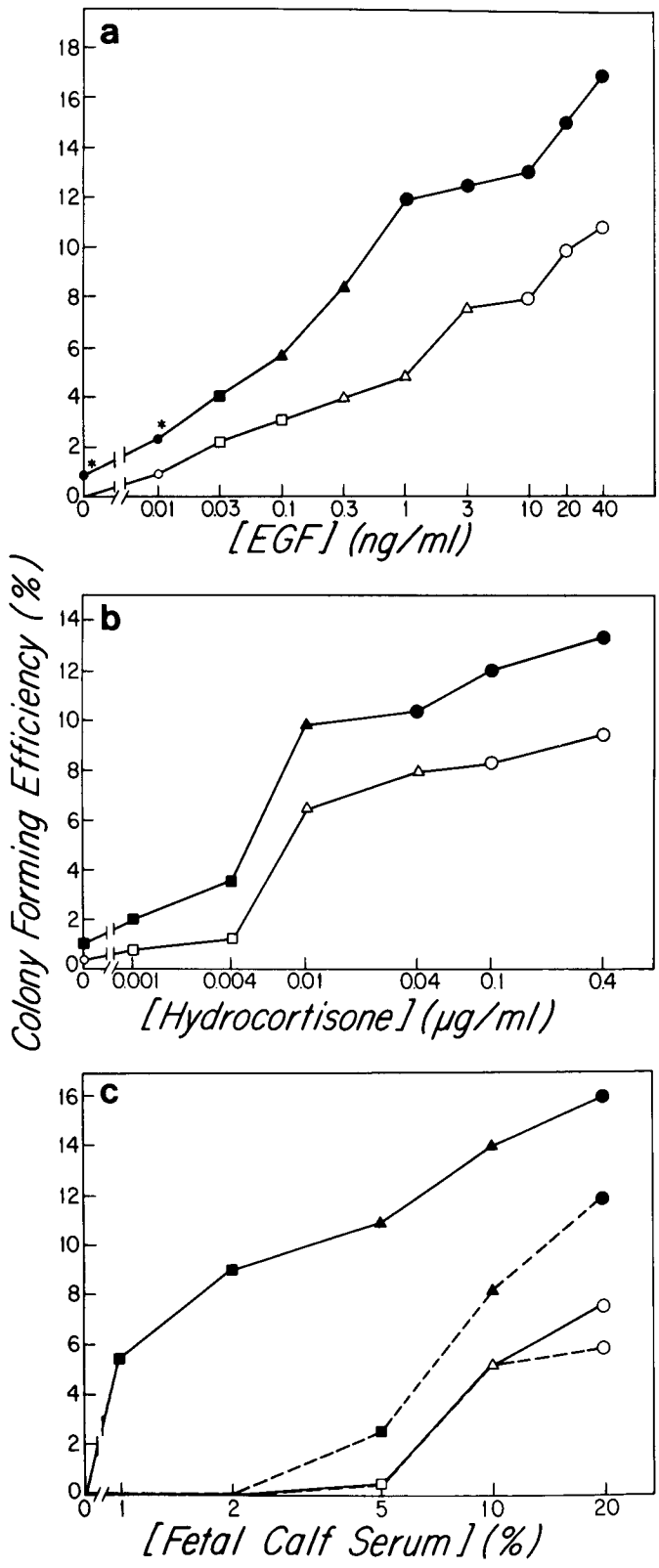
tion of methylcellulose (4000 centipoise, Fisher Scientific, Medford, MA) to a final concentration of 1.3%, using a modification (25) of a method first described by Stoker (31).



Assessment of clonal growth. Cultures were scored for colony forming efficiency and average colony size ten days after plating—a time at which colonies growing in semi-solid medium had reached their maximum size. Colony size and colony forming efficiency (CFE) in surface culture were estimated by examination of methylene blue-stained dishes under a dissecting microscope. CFE in semi-solid medium was estimated by counting the number of colonies in eight random fields as observed through the inverted microscope and relating the area of the magnified visual field to the surface area of the dish. Colony diameters in semi-solid medium were measured using an eyepiece fitted with a calibrated reticule.

Preparation and antibody-neutralization of biological fluids. Freshly collected human urine was filter-sterilized through an 0.2μ filter (Millex-GV, Millipore, Bedford, MA) and diluted into the semi-solid medium. Blood was collected and pooled from the thoracic and abdominal vessels of ether-anesthetized male, Balb/c nude mice, and was centrifuged in the cold. The plasma was filter-sterilized as above and used between 1-6 hours after collection. Blood was collected without anesthesia from the forearm of a healthy adult male and was processed the same way.

Dilutions of freshly obtained male mouse plasma or of receptor grade EGF (Collaborative Research, Lexington, MA) were preincubated with 1.5 or 5 μg affinity-purified anti-mouse EGF (Seragen, Boston, MA) at 37° for 30 minutes, and the mixtures were then diluted 1:100 into 10 ml of semi-solid medium immediately before adding the cells.

Results

We first compared the growth of mesothelial cells in surface culture and in semi-solid medium in response to a broad range of EGF concentrations (Figure 1a). The concentration response for EGF was similar for growth under both conditions, except for one difference: in the absence of EGF, very slow (doubling time~3d) but progressive growth occurred in surface culture whereas no cell division took place in semi-solid medium, even in the presence of 20% fetal calf serum (FCS). (Ten different serum lots from four different commercial suppliers were tested.) Small colonies grew in semi-solid medium in response to the addition of as little as 10-30 pg/ml EGF.

FIG. 1. Growth factor-dependence of normal human mesothelial cells in semi-solid medium and in surface culture. Early passage LP-9 cells (15-25 cell generations) were seeded into bacteriological petri dishes (Falcon) at 10^5cells/10 ml of medium containing 1.3% methylcellulose or onto tissue culture dishes at 300 cells/5 ml of regular medium. Average colony size in surface culture: (●)>500 cells; (▲) 200-500 cells; (■) 75-200 cells; (•) 25-75 cells. Colony size (diameter) in semi-solid medium: (○) 0.5-0.75 mm; (△) 0.3-0.5 mm; (□) 0.1-0.3 mm; (○) 6-8 cells. *a*, Growth in M199/MCDB202 + 20% FCS + 0.4 μg/ml HC + varied concentrations of EGF. *b*, Growth in M199/MCDB202 + 20% FCS + 10 ng/ml EGF + varied concentrations of HC. *c*, Growth in either M199 (--) or M199/MCDB202 (—) supplemented with 0.4 μg/ml HC + 10 ng/ml EGF + varied concentrations of FCS.

In complete medium with 10 ng/ml or more EGF, colonies formed at an efficiency of as much as 10% and reached a maximum diameter of 0.7 mm (several hundred cells). No growth occurred in semi-solid medium in the absence of EGF even if the serum concentration was raised to 30% and the HC to 4 μg/ml, nor was the growth response to low concentrations of EGF enhanced by increased serum or HC concentrations (data not shown). Thus mesothelial cells are exquisitely sensitive to EGF, responding to concentrations that are near the limit of detection by radioimmunoassay (10,30) and below the limit of radioreceptor assay (18). Because stimulation by this small amount of EGF was detected in the presence of 20% fetal calf serum, whole serum must contain less than about 0.1 ng/ml biologically active EGF.

We next compared the growth of mesothelial cells in surface culture and semi-solid medium over a range of HC concentrations (Figure 1b). Growth was attenuated by reducing HC, but some growth still occurred in the absence of added HC. HC is a stable component of fetal calf serum and is usually present at concentrations of 1 to 30 ng/ml, according to the supplier (Sterile Systems, Logan, UT). The serum lot we used for this experiment was assayed at 23 ng/ml HC. Thus the 20% serum supplement provided 4.6 ng/ml HC, which might explain the amount of background growth observed. On the other hand, we do not know whether the requirement satisfied by HC is specific for HC or whether, instead, the serum is providing another steroid or an altogether different compound.

Figure 1c illustrates the effect of limiting serum on mesothelial cell growth. In M199, the serum requirements for surface and anchorage-independent growth were similar. The addition of MCDB202 (12,13), a carefully optimized nutrient medium permitting growth of many cell types in low serum, substantially reduced the serum requirement for mesothelial cell growth in surface culture (Fig. 1c). Nevertheless, little growth occurred in semi-solid M199/MCDB202 with less than 5% serum. Even when EGF was increased to 50 ng/ml and HC to 4 μg/ml, the serum requirement for anchorage-independent growth did not decrease (data not shown). The high serum requirement for anchorage-independent growth in M199/MCDB202 is probably due to a requirement for serum TGF-β (8), which anchored cells do not require for growth.

We then sought to determine whether growth promotion of mesothelial cells in semi-solid medium could be used as a bioassay for EGF activity in biological fluids. We examined urine, which has been found to have a high concentration of EGF (10,16,30), and plasma, which has been reported to have either very low levels or no EGF (14,15,16,22). We compared the growth of mesothelial cells in response to different amounts of human urine added to culture medium containing optimal concentrations of serum and hydrocortisone, but no added EGF (Figure 2a). The mesothelial growth assay measured about 135 ng/ml EGF activity in human urine, which is similar to that determined by radioimmunoassay (10,16,30).

We then assayed EGF activity in freshly isolated human and mouse plasma. We detected no EGF activity in several preparations of plasma withdrawn from a normal adult male (data not shown), consistent with the estimate of ≤0.1 ng/ml obtained by radioimmunoassay of EGF quantitatively extracted by immunoadsorption from human plasma (16,22). In contrast, we detected substantial EGF activity in plasma obtained from adult male mice (Figure 2b). Our value of 135 ng/ml was much higher than that measured in normal mouse plasma by radioimmun-

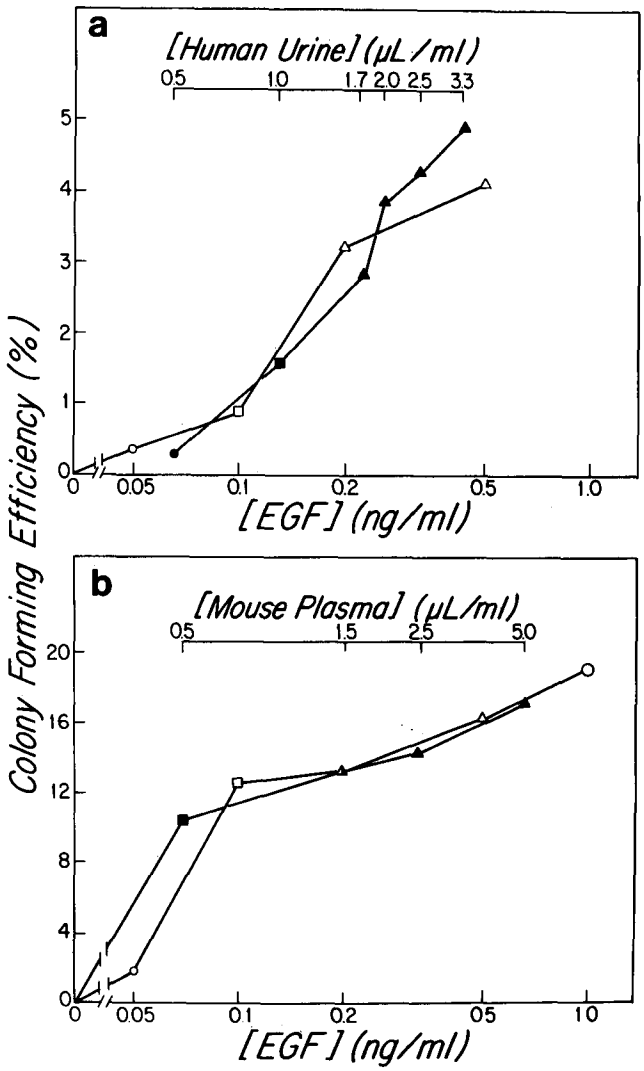


FIG. 2. Estimation of EGF activity in human urine and mouse plasma. LP-9 mesothelial cells were seeded at 10^5cells/10 ml in M199/MCDB202 containing 1.3% methylcellulose, supplemented with 20% FCS and 0.4 μg/ml HC plus either EGF or samples of urine or plasma. Symbols for colony growth are as indicated for semi-solid medium in Figure 1. Open symbols indicate mouse EGF standards, and closed symbols indicate biological fluid assayed (*a* human urine, *b* mouse plasma). Best-fit curves were generated by laterally adjusting the position of the y axis of the urine or plasma concentrations tested so that the responses would best correspond with those of the EGF standard curve in each experiment. (The relatively low %CFE was due to the higher passage level of the cells used in experiment *a*.)

noassay (5,6). However, Byyny and colleagues (5) also found that epinephrine administered to male mice before bleeding caused an increase in plasma EGF to as much as 100 ng/ml. Our method of obtaining blood after lethal ether anesthesia or cervical dislocation might have resulted in epinephrine release, thus accounting for the high EGF levels we observed. In one experiment comparing growth stimulation by male and female mouse plasma, female plasma had substantially less EGF activity than male plasma (data not shown).

To ensure that the mesothelial growth stimulatory activity of mouse plasma measured in this system was EGF and not another factor, we sought to block EGF activity specifically by preincubation with anti-mouse EGF antiserum. Affinity-purified anti-mouse EGF (Seragen, Boston, MA) was preincubated at a 3- to 100-fold molar excess with pure mouse EGF (Figure 3a) or with mouse plasma (Figure 3b), and the mixture was then added to the cells and culture medium. Antibody preincubation decreased the colony formation stimulated by mouse plasma or by purified EGF. The inhibitory effect of the anti-EGF was largely mitigated by higher EGF or plasma concentrations, but the antibody virtually extinguished the growth-promoting effect of lower levels of plasma or EGF, thus demonstrating that essentially all the growth promoting activity measured in mouse plasma was due to EGF.

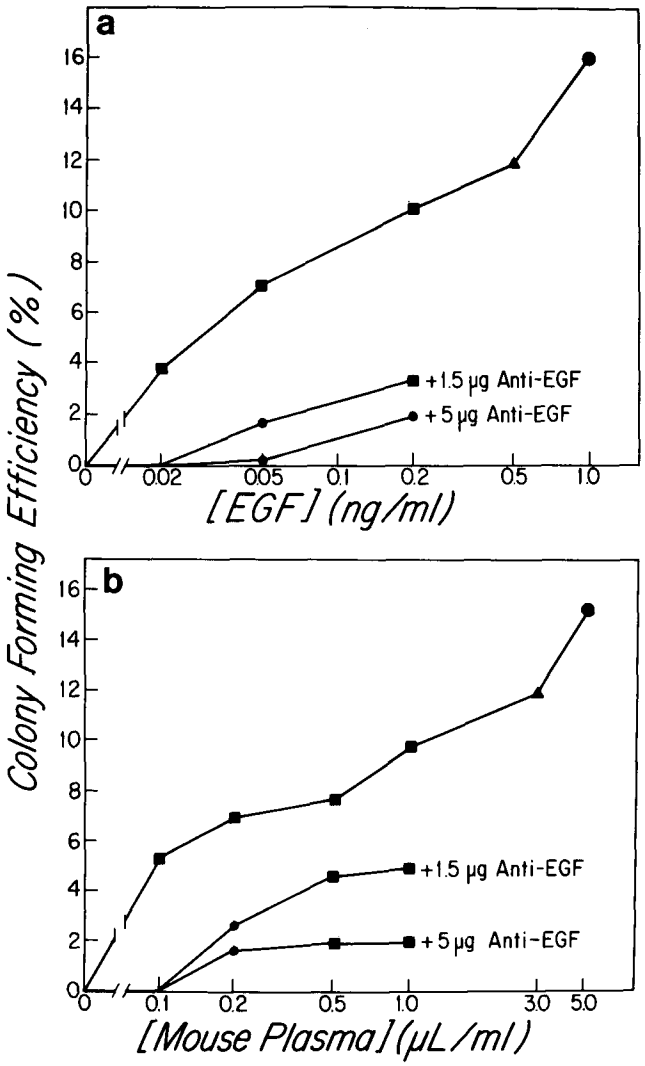


FIG. 3. Neutralization of EGF bioactivity in mouse EGF standards and fresh mouse plasma by anti-EGF antiserum. Mesothelial cells were seeded at 10^5cells/10 ml in M199/MCDB202 containing 1.3% methylcellulose supplemented with 20% FCS and 0.4 μg/ml HC plus (*a*) EGF (Receptor grade, Collaborative Research, Lexington, MA) or (*b*) fresh mouse plasma (prepared as described in Figure 2). Some samples in *a* and *b* were preincubated with 1.5 or 5 μg affinity-purified anti-mouse EGF (Seragen) at 37° for 30 min, and then were diluted 1:100 into 10 ml of semi-solid medium with cells at the time of plating. Symbols for colony growth are as indicated for semi-solid medium in Figure 1.

DISCUSSION

The contribution of EGF and HC specifically to anchorage-independent growth of normal human mesothelial cells is not easily determined, as it has been for normal human fibroblasts. This is because both agents are required for optimal growth of mesothelial cells in surface culture, as well (9,27). EGF and HC have at most a modest stimulatory effect on normal human fibroblasts growing in surface culture in the presence of high (10-20%) serum. Peehl and Stanbridge (23) observed a strong stimulatory effect of HC and no effect of EGF on human fibroblasts for growth in semi-solid medium, and we have confirmed these results (data not shown). EGF stimulates, but is not absolutely required for, anchorage-independent growth of normal bovine granulosa cells (3) and NRK cells (17), consistent with a synergism between EGF and TGF-β (1). In spite of the fact that normal mesothelial cells require EGF for optimal growth under all conditions, they can grow very slowly in surface culture in the absence of EGF (9,32). That EGF plays a specific role in anchorage-independent growth of mesothelial cells is suggested by the fact that they cannot divide at all in semi-solid medium in its absence.

The response of mesothelial cells in this system to the addition of as little as 30 pg/ml EGF in the presence of 20% fetal calf serum demonstrates that there is less than about 0.1 ng/ml of biologically active EGF in whole serum. This estimate is similar to the measurement of immunoreactive EGF in human serum by Hirata, Orth and colleagues (14,15,16). There is, therefore, less than 10 pg/ml EGF in culture medium supplemented with 10% serum.

It seems paradoxical that mitogenic levels of EGF are lacking in the serum supplement of traditional cell culture media, whereas EGF at a concentration of 1-10 ng/ml is found to be an important mitogen for many cell types in "defined media" lacking serum (2,4). Cells may develop an additional requirement for EGF stimulation in order to grow in the absence of certain serum components. Alternatively, for some cells EGF may be able to functionally replace the factors in serum that normally stimulate growth in culture. Thrombin has been reported

to largely compensate for the absence of EGF in a serum-free medium for hamster fibroblasts (7). EGF is likely to serve a different function for epithelial cell types that are dependent upon this factor for growth in culture even in the presence of high concentrations of serum (for example, see 9,26,27).

The recent report by Oka and Orth (22) that immunoreactive EGF is not present in human plasma, but rather is released from platelets during the clotting process, suggests that EGF is only required by many cells in vivo for growth during wound repair. Perhaps the pericellular environment during wound repair in vivo has a feature in common with semi-solid medium and (for many epithelial cell types) tissue culture plastic - namely, the absence of certain substratum components present in the normal extracellular matrix. EGF may provide some cell types with a mitogenic signal that helps to bypass a requirement for contact with certain substratum components.

Lastly, the sensitivity of mesothelial cells to as little as 30 pg/ml added EGF demonstrates that mesothelial cell growth promotion is a more sensitive assay for EGF than the radioreceptor assay (18), and is as sensitive as the radioimmunoassay (6,10,30). Unlike the radioimmunoassay, the bioassay we have described here is not adversely affected by high concentrations of serum protein. Because mesothelial cells detect EGF-like biological activity, rather than EGF immunological cross-reactivity, the human mesothelial cell/semi-solid medium system can distinguish biologically active from inactive forms of EGF. Thus it has the potential of identifying other compounds or polypeptides that have the same biological effect as EGF.

REFERENCES

1. Anzano, M. A.; Roberts, A. B.; Meyers, C. A.; Komoriya, A.; Lamb, L. C.; Smith, J. M.; Sporn, M. B. Synergistic interaction of two classes of transforming growth factors from murine sarcoma cells. Cancer Res. 42: 4776-4778; 1982.
2. Barnes, D.; Sato, G. Methods for growth of cultured cells in serum-free medium. Anal. Biochem. 102: 255-270; 1980.
3. Bertoncello, I.; Bradley, T. R.; Chamley, W. A.; Hodgson, G. S. The characteristics of an anchorage-independent clonal agar assay for primary explanted bovine granulosa cells. J. Cell Phys. 113: 224-230; 1982.
4. Bettger, W. J.; Boyce, S. T.; Walthall, B. J.; Ham, R. G. Rapid clonal growth and serial passage of human diploid fibroblasts in a lipid-enriched synthetic medium supplemented with epidermal growth factor, insulin, and dexamethasone. Proc. Natl. Acad. Sci., USA 78: 5588-5592; 1981.
5. Byyny, R. L.; Orth, D. N.; Cohen, S.; Doyne, E. S. Epidermal growth factor: Effects of androgens and adrenergic agents. Endocrinol. 95: 776-782; 1974.
6. Carpenter, G.; Cohen, S. Epidermal growth factor. Ann. Rev. Biochem. 48: 193-216; 1979.
7. Cherington, P. V.; Pardee, A. B. Synergistic effects of epidermal growth factor and thrombin on the growth stimulation of diploid Chinese hamster fibroblasts. J. Cell Physiol. 105: 25-35; 1980.
8. Childs, C. B.; Proper, J. A.; Tucker, R. F.; Moses, H. L. Serum contains a platelet-derived transforming growth factor. Proc. Natl. Acad. Sci., USA 79: 5312-5316; 1982.
9. Connell, N. D.; Rheinwald, J. G. Regulation of the cytoskeliton in mesothelial cells: Reversible loss of keratin and increase in vimentin during rapid growth in culture. Cell 34: 245-253; 1983.
10. Dailey, G. E.; Kraus, J. W.; Orth, D. N. Homologous radioimmunoassay for human epidermal growth factor (Urogastrone). J. Clin. Endocrinol. Metab. 46: 929-936; 1978.
11. DeLarco, J. E.; Todaro, G. J. Growth factor from murine sarcoma virus-transformed cells. Proc. Natl. Acad. Sci. USA 75:4001-4005; 1978.
12. Ham, R. G. Media and growth requirements. Pastan, I.; Jakoby, W., eds. Methods in Enzymology, Vol. 58. New York: Academic Press; 1980: 55-72.
13. Ham, R. G.; McKeehan, W. L. Development of improved media and culture conditions for clonal growth of normal diploid cells. In Vitro 14: 11-22; 1978.
14. Hirata, Y.; Moore, G. W.; Bertagna, C.; Orth, D. N. Plasma concentrations of immunoreactive human epidermal growth factor (Urogastrone) in man. J. Clin. Endocrinol. Metab. 50: 440-444; 1980.
15. Hirata, Y.; Orth, D. N. Epidermal growth factor (Urogastrone) in human tissues. J. Clin. Endocrinol. Metab. 48: 667-672; 1979.
16. Hirata, Y.; Orth, D. N. Epidermal growth factor (Urogastrone) in human fluids: Size heterogeneity. J. Clin. Endocrinol. Metab. 48: 673-679; 1979.
17. Jetten, A. M.; Goldfarb, R. H. Action of epidermal growth factor and retinoids on anchorage-dependent and -independent growth of non-transformed rat kidney cells. Cancer Res. 43: 2094-2099; 1983.
18. Ladda, R. L.; Bullock, L. P.; Gianopoulos, T.; McCormick, L. Radioreceptor assay for epidermal growth factor. Anal. Biochem. 93: 286-294; 1979.
19. Laug, W. E.; Tokes, Z. A.; Benedict, W. F.; Sargents, N. Anchorage-independent growth and plasminogen activator production by bovine endothelial cells. J. Cell Biol. 84:281-293; 1980.
20. MacPherson, I.; Montagnier, L. Agar suspension culture for the selective assay of cells transformed by polyoma virus. Virology 23: 291-294; 1964.
21. Marquardt, H.; Todaro, G. J. Human transforming growth factor. Production by a melanoma cell line, purification, and initial characterization. J. Biol. Chem. 257: 5200-5225; 1982.
22. Oka, Y.; Orth, D. Human plasma epidermal growth factor/β-Urogastrone is associated with blood platelets. J. Clin. Invest. 72: 249-259; 1983.
23. Peehl, D. M.; Stanbridge, E. J. Anchorage-independent growth of normal human fibroblasts. Proc. Natl. Acad. Sci. USA 78: 3053-3057; 1981.
24. Rapp, U. R.; Gunnell, M.; Marquardt, H. Normal mouse serum contains peptides which induce fibroblasts to grow in soft agar. J. Cell. Biochem. 21: 29-38; 1983.
25. Rheinwald, J. G.; Green, H. Growth of cultured mammalian cells on secondary glucose sources. Cell 2: 287-293; 1974.
26. Rheinwald, J. G.; Green H. Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes. Nature 265: 421-424; 1977.
27. Rheinwald, J. G.; O'Connell, T. M.; Connell, N. D.; Rybak, S. M.; Allen-Hoffmann, B. L.; La Rocca, P. J.; Wu, Y.-J.; Rehwoldt, S. M. Expression of specific keratin subsets and vimentin in normal human epithelial cells - a function of cell type and conditions of growth during serial culture. In: Cancer Cells/The Transformed Phenotype. New York: Cold Spring Harbor Laboratory; 1984:217-227.
28. Roberts, A. B.; Lamb, L. C.; Newton, D. L.; Sporn, M. B.; De Larco, J. E.; Todaro, G. J. Transforming growth factors: Isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extraction. Proc. Natl. Acad. Sci. USA 77: 3494-3498; 1980.

29. Shin, S.-I.; Freedman, V. H.; Risser, R.; Pollack, R. Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage independent growth *in vitro*. Proc. Natl. Acad. Sci. USA 72: 4435-4439; 1975.
30. Starkey, R. H.; Orth, D. N. Radioimmunoassay of human epidermal growth factor (Urogastrone). J. Clin. Endocrinol. Metab. 45: 1144-1153; 1977.
31. Stoker, M. Abortive transformation by polyoma virus. Nature 218: 234-238; 1968.
32. Wu, Y.-J.; Parker, L. M.; Binder, N. E.; Beckett, M. A.; Sinard, J. H.; Griffiths, C. T.; Rheinwald, J. G. The mesothelial keratins: A new family of cytoskeletal proteins identified in cultured mesothelial cells and non-keratinizing epithelia. Cell 31: 693-703; 1982.
33. Horwitz, A. L.; Dorfman, A. The growth of cartilage cells in soft agar and liquid suspension. J. Cell Biol. 45: 434-438; 1970.

We thank Ms. Susan Rehwoldt for excellent technical assistance and Ms. Heather Ferguson for preparing the manuscript. This work was supported by NIH grants RO1 AG02048 and RO1 CA26656 to James G. Rheinwald and by NIH postdoctoral fellowship F32 AG05303 to Paul J. La Rocca.