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Cultivation, Serial Transfer, and Differentiation of Epidermal Keratinocytes in Serum-Free Medium

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We describe serum-free culture conditions for human epidermal keratinocytes using lethally treated 3T3 cells as feeder layers and normal Ca^{++} concentrations (1.2 mM), in a DMEM/F12-Ham nutrient mixture supplemented with several additives, and 10 mg/ml bovine serum albumin instead of animal serum. Keratinocytes were serially grown to 15–18 cell generations (4 subcultivations) and formed a stratified squamous epithelium that could be detached as a graftable epithelial sheet. EGF and TGFα significantly increased keratinocyte proliferation under these conditions; EGF reduced the expression of keratin K1, which is specific for stratified and terminally differentiated epidermal keratinocytes. In contrast with previous reports, the serum-free medium we describe here supports serial growth and normal differentiation of human epidermal keratinocytes, and the formation of graftable stratified epithelia; it also supports the assay of a variety of cytokines or compounds that modulate epidermal keratinocyte proliferation and differentiation.

The growth of many cell types in culture requires serum in the medium. Due to the complexity and undefined nature of serum, investigators in the field have attempted to grow cells under culture conditions without serum. A serum-free medium for diploid keratinocyte cultivation should support their serial growth and normal differentiation, and make possible to study the effects of cytokines or drugs on proliferation and expresion of differentiation markers in the absence of serum components that may bind, inactivate, antagonize, or mimic the action of those agents to be studied.

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Abbreviations: EGF, epidermal growth factor; TGFα, Transforming growth factor-α; SFM, serum-free medium; FBS, fetal bovine serum; HEK, human epidermal keratinocytes.

Previously, human epidermal keratinocytes (HEK) have been cultured in medium in which the serum supplement was eliminated by addition of crude bovine hypothalamic extract (BPE) and trace elements (1, 2). Later, media based on the MCDB-153 formulation were also developed (3-5). In all cases BPE, a highly complex mixture of proteins and growth factors (1, 6), was used as supplement. In addition to BPE, serum-free media for HEK cultivation required plating at high cell densities, low Ca^{++} concentrations (0.2-0.3mM) (1-5) to support about 4-8 subcultivations of epidermal keratinocytes (1, 3, 5, 7); normal differentiation and epithelial stratification were absent (8, 9), since keratin K1 expression was lacking (8), involucrin was expressed in basal cells (10) and abnormal intracellular actin microfilament structure was seen (8) in low Ca++ concentrations. Other authors have reported that EGF was not mitogenic for human keratinocytes cultured in SFM (11). All these alterations in the proliferation and differentiation of cultured keratinocytes limit the usefulness of such culture serum-free media conditions.

Here, we describe serum-free culture conditions for HEK with lethally treated 3T3 feeder layers and with normal Ca^{++} concentrations (1.2 mM). The HEK were serially grown to 4 subcultivations (15-18 generations), they expressed normal differentiation markers, and their response to growth factors was easily assayed. They formed a graftable epithelium with well organized 4-5 layered tissue, with distinctive basal cell layer and flattened suprabasal cells. Keratin K1, a terminal differentiation marker of epidermal keratinocytes, showed the normal decreased level of expression in the presence of EGF. Cultivation in serum-free media of epidermal sheets for grafting in various skin diseases will overcome some of the raised concerns by the use of the serum supplement.

MATERIALS AND METHODS

Materials. HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]), bovine serum albumin (BSA, cat # A8022, minimal

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purity 96%), insulin, hydrocortisone, human transferrin, cholera toxin, L-triiodothyronine (L-T_3), mitomycin C, Rhodamine B and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO). Ham's F12 nutrient mixture and Eagle's medium modified by Dulbecco-Vögt (DMEM) were purchased from Life Technologies (Gaithersburg, MD). Cultispher-GL collagen microcarriers and fetal bovine serum (FBS) were purchased from HyClone Laboratories (Logan, UT). Dispase II was from Boehringer Mannheim Biochemica GmbH (Mannheim, Germany). The recombinant human Transforming Growth Factor-α (TGFα) was from R & D Systems (Minneapolis, MN), and the recombinant human Epidermal Growth Factor (EGF) from Mallinckrodt Specialty Chemicals Co. (Chesterfield, MO). Prestained molecular weight markers were from Bio-Rad (Hercules, CA). All other reagents used were analytical grade.

Cell culture. The 3T3 fibroblasts were used as feeder layers (12, 13). The 3T3 cells were cultured in large quantities in suspension cultures on collagen microcarriers; they were frozen in liquid nitrogen at high cell concentrations (between 20×10^6 to 100×10^6 cells per ml) with medium containing 10% (v/v) glycerol or 10% (v/v) DMSO and 10% (v/v) calf serum. Cells were lethally treated with 4 μg/ ml mitomycin C for 2 hours before freezing, or in some cases, mitomycin C treatment was done after cells were thawed. The HEK (strain HE-123 and strain HE-132) were obtained from newborn foreskin and cultured as previously described (12,14). Disaggregated keratinocytes (2.7×10^3cells/cm^2) were mixed with mitomycin C-treated 3T3 cells (2.2×10^4 cells/cm^2) (12,15), and seeded using a (3:1) DMEM/ F12-Ham nutrient mixture supplemented with 10% (v/v) FBS, plus 5.0μg/ml insulin, 5.0μg/ml transferrin, 0.4μg/ml hydrocortisone, 2×10^{-9}M L-T_3, 1×10^{-10}M cholera toxin, and 24.3 mg/l adenine. After 10 days, primary cultures were dissagregated with trypsin and cells were frozen. For all experiments, keratinocytes were thawed and plated at 2.7×10^3 cells/cm^2 together with mitomycin C-treated 3T3 cells (see above) in medium containing 10%(v/v) FBS. One day after plating, cells were rinsed three times with serum-free DMEM and changed to a DMEM/F12-Ham (3:1) nutrient mixture containing same supplements as above and 30 mM HEPES pH 7.4, 10 ng/ml EGF, and 10 mg/ml BSA instead of serum (serum-free medium, SFM); control cultures were supplemented with 10% FBS. HEK were subcultured 7 days after seeding and medium was changed every 3 days. To assay for growth factor activity, cultures were seeded at 6.75×10^2cells/cm^2; one day after plating, they were washed three times with DMEM, cultured with SFM and the indicated concentrations of growth factors. Keratins were extracted as previously described (16, 17).

For colony forming efficiency (CFE) after each transfer, keratinocytes (strain HE123 or HE132) were plated in indicator dishes at 1×10^3 cells/60-mm dish together with mitomycin C-treated 3T3 cells, in medium containing 10% FBS; cultures were incubated for 10 days, and medium was changed every 3 days. Cultures were fixed, stained with Rhodamine B and CFE values and proportion of aborted colonies were determined. Aborted colonies have a short replicative potential, and they were described as paraclones (18).

RESULTS

Serial growth of HEK in serum-free medium. Primary frozen HEK were thawed, seeded and subcultured as described in Methods. Serial transfer of HEK was carried out as many times as possible and the number of population doublings was calculated after each transfer. Keratinocytes grown in SFM or in medium containing serum were serially transferred, and by the 4th subcultivation growth rate decreased due to senescense. Number of generations obtained after each transfer was similar for both cell culture conditions

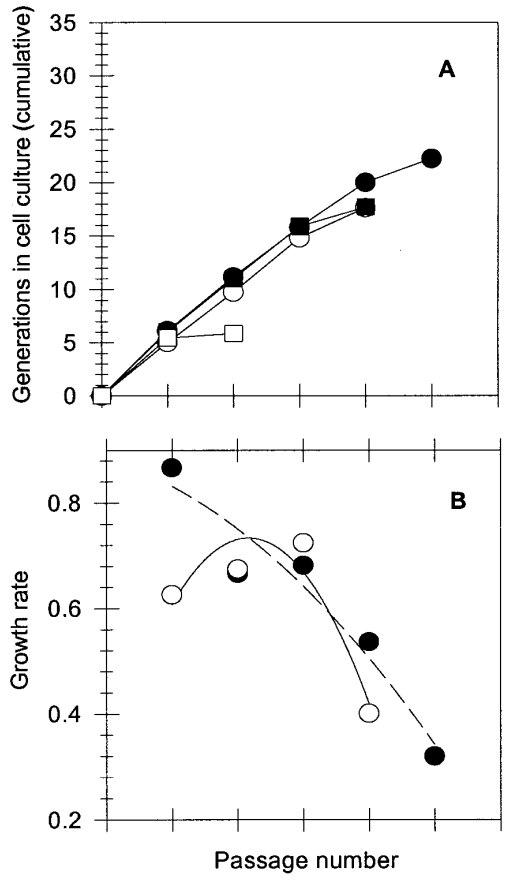

FIG. 1. Serial transfer of HEK grown under serum-free culture conditions. (A) Strains HE123 (■, □) and HE132 (●) cultured with serum-free medium containing (■, ●) or not (□) 10 ng/ml EGF. Strain HE123 fed with serum supplemented medium (○). (B) Growth rates of HEK grown in SFM (--●--) or medium supplemented with serum (○). Growth rate was calculated as the ratio between cell generations and days elapsed in cell culture after each transfer.

(Fig. 1A), and those cells grown in SFM showed similar growth rates as keratinocytes supplemented with serum (Fig. 1B). It should be remarked that keratinocyte growth was dependent upon growth factor addition since those cells cultured without EGF could not be transferred more than one passage (see Fig. 1A).

Cultured keratinocytes form colonies with the proliferating and migrating cells located at the edge of the growing colony (19). Figure 2 (A, B) shows that cultivation of keratinocytes with serum-free medium led to formation of colonies with morphology similar to that observed in medium containing serum; colonies were round and well spread with proliferating and migrating cells at the colony edge (Figure 2A, B); in both culture conditions, some of the colonies showed stratification beginning at the center. In other experiments, 3-day post-confluent epithelia obtained by cultivation of epidermal keratinocytes, with or without serum, were detached from culture dishes with 2.5 mg/ml Dispase (20) and fixed for histological examination. Epithelia ob-

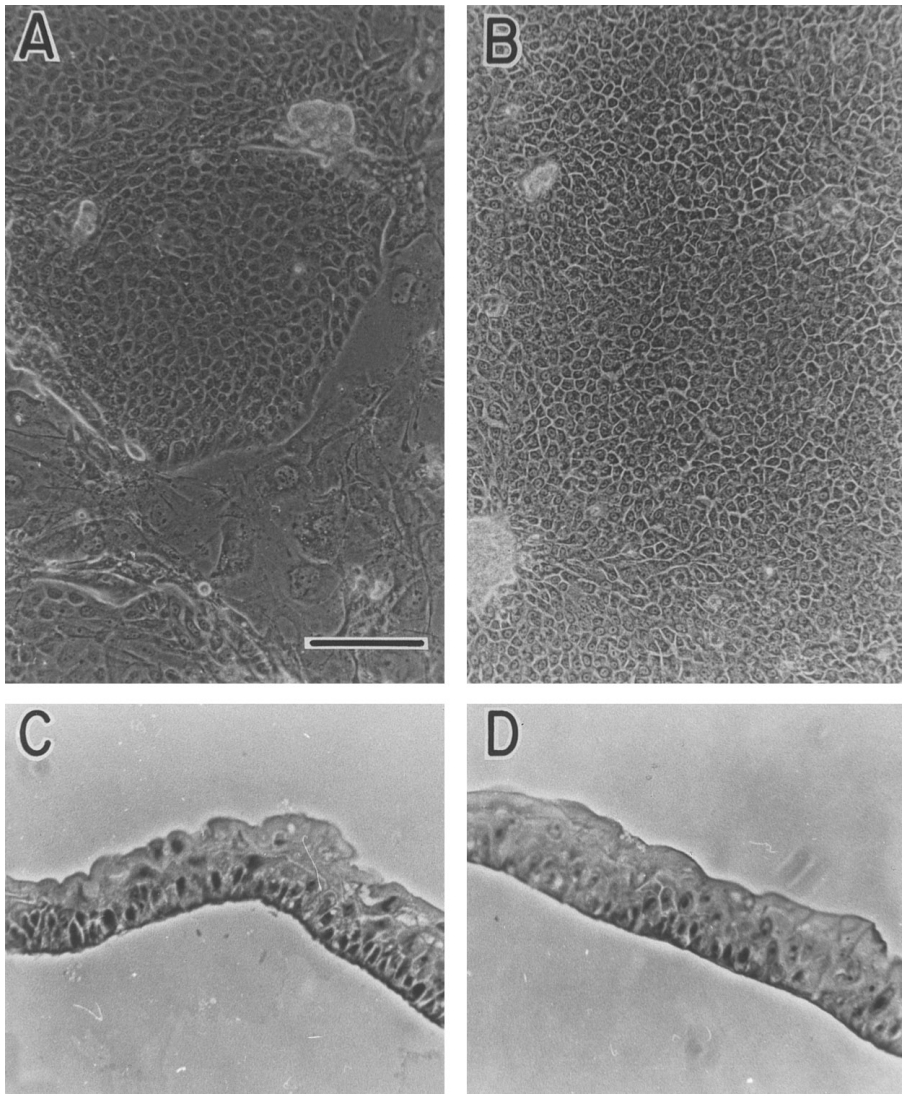

FIG. 2. Morphology of keratinocytes cultured under SFM conditions. (A) An early colony of cells cultured in the presence of 10 ng/ml EGF. (B) Confluent epithelium obtained after 8 days in cell culture. Bar=150 μm. Transversal sections of 3-day post-confluent epithelia grown under medium containing serum (C), or SFM culture conditions (D).

tained by cultivation under SFM conditions consisted of a well organized 4-5 layered tissue, with a distinctive basal cell layer and flattened suprabasal cells, similar to cultures in serum supplemented medium (Figure 2C, D). The results suggest that epidermal keratinocytes cultured with SFM were able to express their proliferative and differentiation capacities and to form a graftable stratified epithelium.

Human epidermal keratinocytes show three clonal types of multiplying cells that could be distinguished by the morphology and proliferative potential of their progeny (18). Thus, we also assayed colony forming efficiency and the proportion of abortive and terminally differentiated colonies for HEK during serial transfer under SFM or medium with 10% FBS. Keratinocytes were obtained from each subcultivation, plated in indicator dishes at 1.0×10^3 cells/60-mm dish in medium containing 10% FBS and cultured for ten days. As shown in Table 1, HE-123 cells showed colony forming ability of about 15%, and these values decreased with every cell passage until the colony forming ability was close to zero in cells coming from cultures maintained with or without the serum supplement. The colony formation ability showed a larger drop in cultures kept in SFM than in serum supplemented medium, but during the two early subcultivations the proportion of abortive and terminally differentiated colonies was similar in cells cultured with serum supplemented media and in cells cultured in SFM; this proportion was larger in cultures approaching senescence (Table 1).

Response of HEK to growth factors in SFM. HEK were seeded at 6.75×10^2 cells/cm^2 and maintained

TABLE 1

Proportion of Abortive and Terminally Differentiated Colonies in Human Epidermal Keratinocyte Cultures Kept in Serum-Free Medium with EGF

	Serum supplemented		Serum-free	
Subculture	Colony formation ability (%)	Aborted colonies (% of total CFE)	Colony formation ability (%)	Aborted colonies (% of total CFE)
1	15.5 ± 0.76	18.1 ± 0.65	8.73 ± 1.33	17.1 ± 0.22
2	14.4 ± 0.28	34.4 ± 0.45	9.00 ± 0.29	40.4 ± 0.50
3	7.97 ± 0.98	45.2 ± 0.08	1.53 ± 0.46	65.2 ± 0.40
4	5.86 ± 0.37	48.8 ± 0.45	1.00 ± 0.16	60.0 ± 0.10

Note. Keratinocytes (HE123 strain) were serially transferred under SFM conditions or medium with 10% FBS; both culture conditions contained 10 ng/ml EGF as described. Colony forming ability (CFE) values and abortive and terminally differentiated colonies were determined in indicator dishes fed with medium containing 10% fetal bovine serum. Data is the average of at least 4 experiments ± S.D.

with SFM as described in Methods. Various concentrations of EGF and TGFα, were added to the medium. Ten days after, cultures were fixed, stained with rhodamine B and cell growth was evaluated. Growth factors significantly stimulated HEK proliferation (Figure 3). EGF, at concentrations below 10 ng/ml, exerted about 10-fold higher stimulation of cell growth as compared to TGFα. However, at higher and saturating concentrations, both growth factors showed similar stimulatory effects. Each growth factor at the highest concentration tested, exerted about 10-15 fold increase in cell density as compared with cultures without the growth factors (Figure 3).

In other experiments, keratinocytes were seeded at 2.2×10^2 cells/cm^2 and changed to SFM containing or not 10 ng/ml EGF. At different times, keratin expression was analyzed in cell extracts. In cultures maintained in SFM with EGF, keratin K1 (67 kD), which is characteristic of suprabasal differentiated cells, constituted 1.9% of total keratin protein; in cells maintained with SFM without EGF keratin K1 was about 5.1% of total keratin protein (Figure 4). Other keratins did not show any significant difference in their expression with or without EGF (Figure 4). Our results suggest that EGF, as expected, reduced the expression of differentiation-linked keratins and promoted a proliferating phenotype when assayed under the described SFM conditions.

DISCUSSION

Since the use of serum-free media should allow a simpler design and interpretation of experiments, some authors have attempted to adapt cells to media without serum; to combine nutrients to support cell growth and differentiation (21), or to use hormones and some characterized macromolecular fractions as additives (22). Serum-free formulations were described for heteroploid cells such as human colon carcinoma-derived cells (23, 24), A431 cells (25), bronchogenic epidermoid carcinoma cells (26), LLC-PK$_1$ and MDCK cells (27).

The human epidermal keratinocytes have been grown under serum-free culture conditions with low Ca^{++} concentrations (less than 0.2mM) using Medium 199, DMEM/F12 mixtures or MCDB-153, supplemented with BPE (1, 2), and the addition of trace elements and growth factors (3-5). However, like serum, the BPE is a highly complex mixture of proteins and growth factors. Thus, some authors attempted to eliminate BPE as a medium supplement (28-32). Nevertheless, when these serum-free media are used, containing or not BPE, cultivation of epidermal keratinocytes depends upon the maintenance of the low Ca^{++} concentrations; otherwise, cells are unable to grow, they stratify and undergo terminal differentiation (8). When the low Ca^{++} concentrations are maintained and the plating density is high, keratinocytes may be subcultured 4-

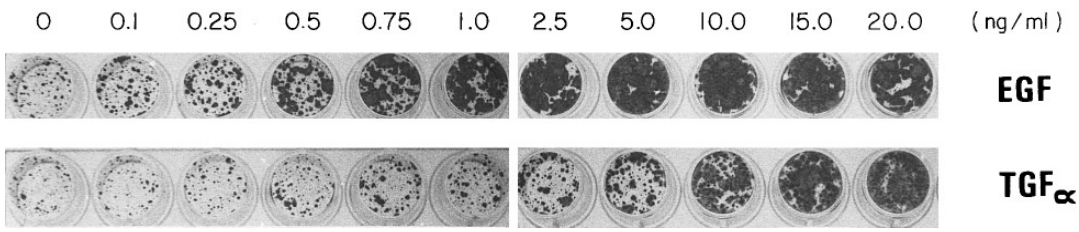

FIG. 3. Response of human epidermal keratinocytes to EGF and TGFα. Cells were maintained in SFM (see Materials and Methods) containing the indicated concentrations of EGF or TGFα. After 14 days, cultures were fixed and then stained with rhodamine B.

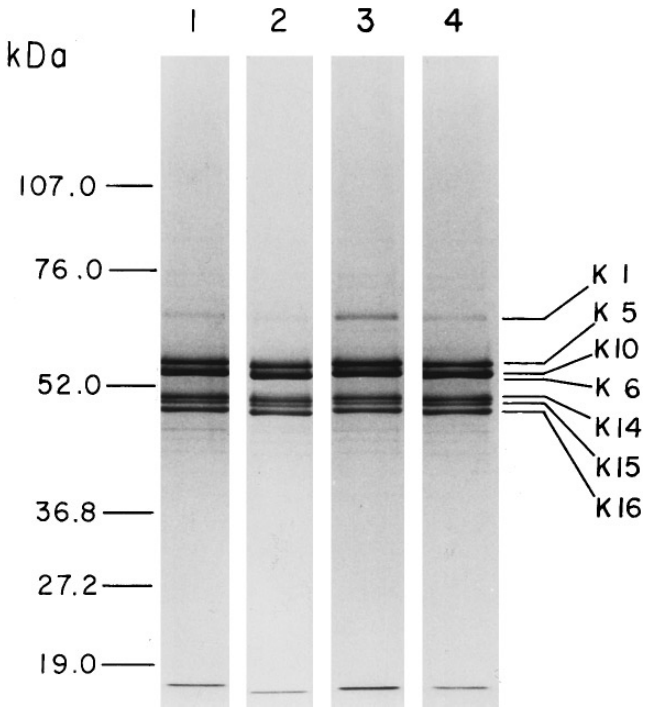

FIG. 4. Changes in keratin expression during cultivation in SFM containing or not EGF. Cells were inoculated in 35-mm dishes as described. One day after, cultures were changed to SFM (lanes 1 and 3); plus EGF 10 ng/ml (lanes 2 and 4). Keratins were extracted after 4 days in culture (lanes 1 and 2), or 3 days after confluence (10 days after inoculation) (lanes 3 and 4). Arrows show the keratin pairs K1/K10, K6/K16, and K5/K14.

8 passages before senescence (5, 7, 30, 33); but they abnormally express, at the basal cell layer, terminal differentiation markers such as involucrin (10). Also, low Ca++ concentrations promote an abnormal intracellular actin microfilament structure (8), and some authors have reported that EGF is not mitogenic for human keratinocytes cultured under these SFM conditions (11).

Hence, we attempted the cultivation of human epidermal keratinocytes with DMEM-F12 medium in the presence of 1.2 mM Ca^{++} and 3T3 feeder cells, and 10 mg/ml bovine serum albumin instead of animal serum. In this case, lethally treated fibroblasts were not only necessary to support keratinocyte growth as described earlier (12, 13), but also allowed the formation of well defined keratinocyte colonies, and the organization of stratified epithelia. Our work is the first to report the cultivation of epidermal keratinocytes under serum-free conditions, at calcium concentrations of about 1.2mM, in which growth, long-term serial transfer, organization of a 4-5 layered epithelium and the normal expression of terminal differentiation markers such as the keratin pair K1/K10 were obtained.

Previously, more than 100 generations in culture were obtained when human epidermal keratinocytes were grown in serum supplemented medium and the presence of 3T3 feeder cells and EGF (34). The cell strains we used underwent about 30 generations of growth before senescence. This lower growth capacity might be due to cell strain differences. In our experience with more than 10 human keratinocyte strains cultured in vitro with serum supplemented media, 3T3 cells and EGF, some of the cell strains show large expansion capability (more than 100 generations) whereas others have shown a more limited proliferative potential (20-25 generations). These differences could be due to yet unknown biological characteristics of the donor cells.

Cultured human keratinocytes show three clonal types of multiplying cells characterized by the morphology and proliferative potential of their progeny; these clones were described as holoclones, meroclones and paraclones (18). Paraclones contain cells with short replicative potential that abort and terminally differentiate (18); and holoclones contain cells with large replicative potential, probably related to cell populations that are part of the stem cell compartment (18). Since the transition from holoclones to the other clonal types during serial cultivation could be affected by culture conditions, we scored the proportion of abortive terminally differentiated colonies as a sensitive assay of the effect of serum-free media on the keratinocyte growth potential. As shown, the proportion of abortive and terminally differentiated colonies was similar in both culture conditions for the early two subcultivations but it increased in cultures approaching senescence in SFM. These results suggested that keratinocyte cultivation in serum-free media at early subcultures does not promote a larger proportion of abortive colonies that would increase the proportion of cells giving rise to aborted and terminally differentiated colonies. These experiments show that cultivation of human epidermal keratinocytes under serum-free conditions containing EGF to promote proliferation, supports serial growth and a similar proportion of proliferating cells able to give rise to non-abortive colonies.

Also, we tested whether SFM conditions allowed to assay for various growth factor activities such as EGF and TGFα. In contrast to others that have reported that EGF is not mitogenic for keratinocytes in SFM (11), our results showed that the SFM is an useful tool to study growth factor effects in cell culture, since the growth factors tested significantly increased cell proliferation of the human epidermal keratinocytes. Moreover, the long-term serial transfer was only possible when 10ng/ml EGF were added; without this growth factor, cultures were limited to one or two cell passages and to about 6 cell generations. Thus, keratinocyte proliferation was dependent upon the addition of the growth factors. On the other hand, EGF and TGFα induced a colony morphology which has been related to a migratory phenotype (19, 35); as expected, these

growth factors led to a decrease on the stratification of cell colonies, and the expression of the terminal differentiation-linked keratin K1 was reduced several fold.

Our results demonstrate that the serum-free conditions described here, should be useful to assay a variety of cytokines, growth factors and compounds that modulate proliferation and differentiation of human epidermal keratinocytes. Recently, human epidermal keratinocytes were cultured at an air-liquid interface onto collagen rafts containing foreskin fibroblasts in a serum-free skin co-culture model (36). Good expression of terminal differentiation markers was seen, but these conditions seemed not to be adequate for serial and clonal growth studies; cell proliferation was only assessed by radioactive thymidine incorporation (36). Our culture system will allow to understand cell growth regulation, since cell proliferation can be analyzed using different parameters. Our culture conditions also supported the formation of confluent cultures that can be detached with Dispase as a stratified squamous epithelium that seems to be suitable as a graftable epidermal sheet. Further modifications to the conditions described here, should allow to develop more optimized serum-free culture conditions that could support a larger expansion for the serial and clonal growth of human epidermal keratinocytes. Since the proliferative potential of isolated single cells is easily assayed in the presence of lethally treated 3T3 feeder layers (18), these serum-free culture conditions will be helpful to characterize stem cells from epithelial tissues and their response to growth factors, cytokines and hormones.

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REFERENCES

1. Maciag, T., Neomore, R. E., Weinstein, R., and Gilchrest, B. A. (1981) *Science* **211,** 1452–1453.
2. Boyce, S. T., and Ham, R. G. (1983) *J. Invest. Dermatol.* **81,** 33s–40s.
3. Pirisi, L., Yasumoto, S., Feller, M., Doniger, J., and DiPaolo, J. A. (1987) *J. Virol.* **61,** 1061–1066.
4. Lechner, J. F., and LaVeck, M. A. (1985) *J. Tiss. Cult. Meth.* **9,** 43–48.
5. Daley, J. P., Epstein, D. A., and Hawley-Nelson, P. (1990) *Focus* **12,** 68–71.
6. Gospodarowicz, D., Bialecki, H., and Greenburg, G. (1978) *J. Biol. Chem* **253,** 3736–3743.
7. Boyce, S. T., and Ham, R. G. (1983) *J. Invest. Dermatol.* **81,** 33s–40s.
8. Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K., and Yuspa, S. H. (1980) *Cell* **19,** 245–254.
9. Hennings, H., and Holbrook, K. A. (1983) *Exp. Cell Res.* **143,** 127–142.
10. Watt, F. M. (1984) *J. Cell Biol.* **98,** 16–21.
11. Hancock, G. E., Kaplan, G., and Cohn, Z. A. (1988) *J. Exp. Med.* **168,** 1395–1402.
12. Rheinwald, J. G., and Green, H. (1975a) *Cell* **6,** 331–344.
13. Rheinwald, J. G., and Green, H. (1975b) *Cell* **6,** 317–330.
14. Bolívar-Flores, J., Poumian, E., Marsch-Moreno, M., Montes de Oca, G., and Kuri-Harcuch, W. (1990) *Burns* **16,** 3–8.
15. Rheinwald, J. G. (1980) *Methods Cell Biol.* **21A,** 229–254.
16. Eichner, R., Bonitz, P., and Sun, T.-T. (1984) *J. Cell Biol.* **98,** 1388–1396.
17. Castro-Muñozledo, F. (1994) *J. Cell Sci.* **107,** 2343–2351.
18. Barrandon, Y., and Green, H. (1987b) *Proc. Natl. Acad. Sci. USA.* **84,** 2302–2306.
19. Barrandon, Y., and Green, H. (1987a) *Cell* **50,** 1131–1137.
20. Green, H., Kehinde, O., Thomas, J. (1979) *Proc. Natl. Acad. Sci. USA.* **76,** 5665–5668.
21. Ham, R. G., and MacKeehan, W. L. (1978) *In Vitro Cell Dev. Biol.* **14,** 11–22.
22. Barnes, D., and Sato, G. (1980) *Anal. Biochem.* **102,** 255–270.
23. Murakami, H., and Masui, H. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 3464–3468.
24. Van der Bosch, J. (1984) *in* Methods for Serum-Free Culture of Epithelial and Fibroblastic Cells (Barnes, D. W., Sirbasku, D. A., and Sato, G. H., Eds.), Vol 3, pp. 73–81, Liss, New York.
25. Barnes, D. W. (1984) *in* Methods for Serum-Free Culture of Epithelial and Fibroblastic Cells (Barnes, D. W., Sirbasku, D. A., and Sato, G. H., Eds.), Vol 3, pp. 95–106, Liss, New York.
26. Miyazaki, K., Masui, H., and Sato, G. H. (1984) *in* Methods for Serum-Free Culture of Epithelial and Fibroblastic Cells (Barnes, D. W., Sirbasku, D. A., and Sato, G. H., Eds.), Vol 3, pp. 83–94, Liss, New York.
27. Saier, M. H. (1984) *in* Methods for Serum-Free Culture of Epithelial and Fibroblastic Cells (Barnes, D. W., Sirbasku, D. A., and Sato, G. H., Eds.), Vol 3, pp. 25–31, Liss, New York.
28. Pillai, S., Bikle, D. D., Hincenbergs, M., and Elias, P. M. (1988) *J. Cell. Physiol.* **134,** 229–237.
29. Tsao, M. C., Walthall, B. J., and Ham, R. G. (1982) *J. Cell. Physiol.* **110,** 219–229.
30. Johnson, E. W., Meunier, S. F., Roy, C. J., and Parenteau, N. L. (1992) *In Vitro Cell. Dev. Biol.* **28A,** 429–435.
31. Peehl, D. M., and Ham, R. H. (1980a) *In Vitro Cell Dev. Biol.* **16,** 516–525.
32. Peehl, D. M., and Ham, R. H. (1980b) *In Vitro Cell Dev. Biol.* **16,** 526–540.
33. Oku, H., Kumamoto, C., Miyagi, T., Hiyane, T., Nagata, J., and Chinen, I. (1994) *In Vitro Cell Dev. Biol.* **30,** 496–503.
34. Rheinwald, J. G., and Green, H. (1977) *Nature* **265,** 421–424.
35. Cha, D., O'Brien, P., O'Toole, E. A., Woodley, D. T., Hudson, L. G. (1996) *J. Invest. Dermatol.* **106,** 590–597.
36. Chen, C-S. J., Lavker, R. M., Rodeck, U., Risse, B., Jensen, P. J. (1995) *J. Invest. Dermatol.* **104,** 107–112.