Stimulation of Thymidine Incorporation in Keratinocytes by Insulin, Epidermal Growth Factor, and Placental Extract: Comparison With Cell Number to Assess Growth

Edward J. O'Keefe, M.D., and Marie L. Chiu, Ph.D.

Department of Dermatology, University of North Carolina, Chapel Hill, North Carolina, U.S.A.

The results of a thymidine incorporation assay were compared with direct measurement of cell number in assessment of proliferative growth of human keratinocytes in monolayer culture. Keratinocytes were cultured in supplemented MCDB 153 medium in 0.1 mM Ca^{2+} , and plated in 24-well trays. The ability of insulin, placental extract, and epidermal growth factor to enhance growth and thymidine incorporation were compared. Autoradiography was performed to determine the percentage of cells with labeled nuclei. Epidermal growth factor increased thymidine incorporation under the conditions of the assay, and placental extract increased incorporation by up to 50-fold, since the control cells plated in the absence of epidermal growth factor and other growth

factors survived but proliferated minimally. Both cell number and thymidine incorporation showed similar concentration dependence upon insulin and placental extract. If placental extract was added to cells plated 28 h earlier, incorporation was maximal after 17 h in the presence of the extract. If cells were plated in the presence of the extract, 85% of nuclei were shown by autoradiography to be labeled after 23 h, but only 24% of nuclei were labeled in the absence of the extract. A plating density of 10^4 cells/2-cm² well was optimal. The assay permits rapid identification of growth-promoting fractions without prolonged growth periods, and is a valid indicator of these agents in keratinocyte cultures.

J Invest Dermatol 90:2-7, 1988

The assessment of keratinocyte growth in vitro is usually performed by direct measurement of growth of plated cells using photography of fixed and stained dishes, cell counts, or measurement of DNA and protein. In contrast, more convenient and rapid assessment of growth using thymidine is conventional in studies of proliferative growth in fibroblasts, lymphocytes, and many other cell types. Several investigators have used labeled nucleosides to perform nuclear labeling in keratinocytes either in vivo [1,2] or in vitro [3]. In vitro, however, when growth of stratified cultures of keratinocytes was assessed using thymidine, parallel measurements of DNA content of the cultures did not correlate with thymidine incorporation [4]. More recently it has become possible to culture keratinocytes as a monolayer by reducing the concentration of calcium ion in the medium to 0.1 mM or less. During studies of the action of insulin, epidermal growth factor (EGF), and placental extract [5] on growth of keratinocytes, we have examined the utility of using nucleoside incorporation as an index of response to growth factors in keratinocytes cultured in a monolayer by comparing the

results of cell counts and thymidine incorporation. The results indicate that, under the conditions of our assay, thymidine incorporation is a valid indicator of proliferative growth in response to growth-promoting agents.

MATERIALS AND METHODS

Cell Culture Keratinocytes were cultured from newborn foreskins according to a modification [6] of the method of Rheinwald and Green [7]. Briefly, foreskins were minced with a scalpel and incubated in trypsin (T 8253, 0.25%; Sigma Chemical Co., St. Louis, MO) and EDTA (0.1% wt/vol) in buffer (trypsin-EDTA buffer) consisting of 30 mM Hepes, 4 mM glucose, 3 mM KCl, 130 mM NaCl, 1 mM sodium phosphate, pH 7.4, and 3.3 μM phenol red [8] for 18 h at 4°C, and the epithelium was removed and dissociated epidermal cells inoculated onto a mitomycin C-treated (4 $\mu\text{g}/\text{ml}$, 2 h, 37°C) 3T3 cell feeder layer in medium consisting of 50% Ham's F-12, 50% Dulbecco's modified minimal essential medium containing 5% fetal bovine serum and supplemented with cholera toxin (10 ng/ml), hydrocortisone (0.4 $\mu\text{g}/\text{ml}$), insulin (5 $\mu\text{g}/\text{ml}$), and transferrin (5 $\mu\text{g}/\text{ml}$). EGF (10 ng/ml) was added after 3 days. After 6-9 days, subconfluent keratinocyte cultures were washed with 0.1% (vol/vol) trypsin (1:250, Gibco, Grand Island, NY) in trypsin-EDTA buffer to remove 3T3 cells, and keratinocytes were suspended with 0.025% trypsin (wt/vol) (T 8253; Sigma Chemical Co.) with 0.01% EDTA (wt/vol) in trypsin-EDTA buffer according to McKeehan et al [8], and inoculated at 10^5 cells/60-mm petri dish into MCDB 153 medium [9] supplemented with EGF (5 ng/ml), insulin (5 $\mu\text{g}/\text{ml}$), hydrocortisone (0.4 $\mu\text{g}/\text{ml}$), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), bovine pituitary extract (150 $\mu\text{g}/\text{ml}$) [9], and six amino acids (histidine, 2.4×10^{-4} M; isoleucine, 7.5×10^{-4} M; methionine, 9×10^{-5} M; phenylalanine, 9×10^{-5} M; tryptophan, 4.5×10^{-5} M; and

Manuscript received January 22, 1987; accepted for publication May 12, 1987.

This work was supported by grant AM-25871 from the National Institutes of Health.

Reprint requests to: E. J. O'Keefe, M.D., 137 NCMH, Department of Dermatology, University of North Carolina, Chapel Hill, North Carolina 27514.

Abbreviations:

- EDTA: ethylenediamine tetraacetic acid
- EGF: epidermal growth factor
- SDS: sodium dodecyl sulfate
- TCA: trichloroacetic acid

tyrosine, 7.5×10^{-5} M), and containing 0.1 mM Ca^{2+} . Cells were carried in this medium and split every 4–6 days according to McKeehan et al [8] and used between passages 2 and 8.

Assay for Growth Using Cell Counts All assays were performed in MCDB 153 medium containing 0.1 mM ethanolamine and phosphoethanolamine, 0.4 μM hydrocortisone, 0.1 mM Ca^{2+} , and six added amino acids, as noted above, and lacking bovine pituitary extract; this is referred to as basal medium. Insulin, EGF, and placental extract were added as described in figure legends. Keratinocytes were plated in duplicate at 10^4 cells/35-mm petri dish (1.25×10^3 cells/cm²) in 3 ml of basal medium. Cells were fed every 3–4 days and counted at indicated intervals in an electronic particle counter (ZBI; Coulter Electronics, Hialeah, FL) after suspension with trypsin and dilution in Isoton (Coulter Electronics). Triplicate counts were performed on each dish, and the counts were averaged.

Thymidine Assay Unless indicated otherwise in the text and figure legends, keratinocytes were plated in 24-well trays (Corning Glass Works, Corning, NY) at 10^4 cells/well (5×10^3 cells/cm²) in 1 ml of MCDB 153 medium supplemented as above (basal medium). Concentration of thymidine was 3 μM . Cultures were treated with or without placental extract, EGF, or insulin in duplicate or triplicate at the time of plating or as noted in legends, and 1 μCi of [³H]thymidine (10 Ci/mmol; ICN, Irvine, CA) was added to each well. After 24 h, cultures were washed 3 times with ice-cold phosphate-buffered saline and twice with cold 5% trichloroacetic acid (TCA) and solubilized with 0.1 N NaOH containing 1% sodium dodecyl sulfate. Solubilized radioactivity was counted in a scintillation counter with Safety Solve (Research Products International, Mount Prospect, IL).

autoradiography Keratinocytes were inoculated into 24-well trays as described above and incubated with [³H]thymidine (1 $\mu\text{Ci}/\text{ml}$) for various times. The incubation was terminated by washing the culture with phosphate-buffered saline at 37°C and then fixing with 2 10-min incubations in absolute methanol. The dried culture was then coated in the well with Kodak nuclear track emulsion NBT-2 (Eastman Kodak Co., Rochester, NY) diluted 1:1 (wt/vol) with distilled water, drained for 10 min, and stored for 3 days in a light-tight dessicator. The film was developed for 10 min in Kodak Microdol X developer, rinsed, and fixed for 3 min with Kodak Rapid Fixer. The cells were counted in the 24-well tray under phase-contrast microscopy to discriminate nuclei with and without silver grains. Two hundred cells were counted per well, and each experimental point was assessed in duplicate.

Materials Chemicals used to make MCDB 153 medium, insulin, hydrocortisone, ethanolamine, phosphoethanolamine, and purified trypsin were from Sigma Chemical Co. EGF was purified to homogeneity according to Savage and Cohen [10]. Placental extract was prepared as previously described [5]. Hepes buffer was from Research Organics (Cleveland, OH).

RESULTS

Comparison of the Effects of Placental Extract or Insulin Using Both Assays To determine whether incorporation of thymidine was a valid indicator of proliferative growth in our cultures, we compared the actions of two agents known to stimulate growth using both cell counts and thymidine incorporation to assess their effects. Insulin is required for growth of keratinocytes in our system at the cell densities used for assessing growth by cell counts (1.25×10^3 cells/cm²) [5], although the requirement for insulin is decreased at the densities of cells used in the thymidine assay (5×10^3 cells/cm²), and is substantially reduced or removed at very high cell density. As shown in Fig. 1a, in the cell counting assay using 10^4 cells per 35-mm dish, minimal growth occurs in the absence of both insulin and placental extract after 8 days, but a slight enhancement occurs if insulin is added, and the concentration of insulin required for half-maximal increase in growth is between 0.5 and 1.0 $\mu\text{g}/\text{ml}$. Similarly, in the thymidine assay performed in parallel in 35-mm

Figure 1. Comparison of response to insulin using cell counting and thymidine incorporation. *A*, Keratinocytes were plated in duplicate 35-mm dishes at 1×10^4 cells per dish in basal medium with or without placental extract (400 $\mu\text{g}/\text{ml}$) in the presence or absence of insulin at various concentrations. Cells were fed every third day, harvested with trypsin after 8 days, and counted in quadruplicate in an electronic particle counter. *B*, Keratinocytes were plated in 24-well trays at 1×10^4 cells per well in basal medium with additions as in (*A*) in triplicate, and 1 μCi of [³H]thymidine was added to each well. After 24 h, incorporation of radioactivity was determined as in *Materials and Methods*. EGF was not added to medium. Solid circles, + placental extract; open circles, - placental extract.

dishes, no incorporation occurs after 24 h in the absence of both insulin and placental extract (Fig 1b), and the increase in incorporation produced by adding insulin to the cultures occurs at lower concentrations, but is half-maximal at less than 1 μg insulin/ml. In the absence of placental extract, therefore, both assays indicate a similar concentration dependence on insulin.

Because negligible thymidine incorporation occurs in the absence of insulin at 24 h even though some growth and incorporation occur if measurements are made several days later, the apparent half-maximal concentration of insulin required in the thymidine assay, about 0.1 $\mu\text{g}/\text{ml}$, is artificially low and is not comparable with that in the cell counting assay, and precise comparison of the half-maximal concentrations is not meaningful. If the initial point from cultures plated in the absence of both insulin and placental extract is ignored and incorporation in the presence of 0.1 μg insulin/ml is used as the zero point for comparison, the half-maximal figure increases to about 0.5 $\mu\text{g}/\text{ml}$ in the thymidine assay, which is similar to the result in the cell counting assay.

If the cells are plated in the presence of placental extract at 400 $\mu\text{g}/\text{ml}$ in the cell counting assay, slight growth occurs after 8

Figure 2. Comparison of response to placental extract using cell counting and thymidine incorporation. *A*, Keratinocytes were plated in duplicate 35-mm dishes at 1×10^4 cells per dish in basal medium with or without insulin ($5 \mu\text{g/ml}$) in the presence or absence of placental extract at various concentrations. Cells were fed every third day, harvested with trypsin after 8 days, and counted in an electronic particle counter. *B*, Keratinocytes were plated in duplicate in 24-well trays at 1×10^4 cells per well in basal medium with additions as in (*A*), and $1 \mu\text{Ci}$ [^3H]thymidine was added to each well. After 24 h, incorporation of radioactivity was determined as in *Materials and Methods*. EGF was not added to medium. Solid circles and solid triangles, + insulin; open circles and open triangles, - insulin.

days in the absence of insulin (5-fold increase in cell number), and insulin stimulates proliferation with a half-maximal effect at about $0.9 \mu\text{g}$ insulin/ml (Fig. 1a). In the case of the thymidine incorporation assay, significant incorporation occurs after 24 h in the absence of insulin, but stimulation is observed in the presence of insulin and is half-maximal at about $0.5 \mu\text{g}$ insulin/ml. Therefore, in the presence of placental extract, as in the absence of placental extract, both assays show a similar dependence on insulin; the concentrations required for half-maximal stimulation are quite similar. The assays are not strictly comparable, since in the absence of both placental extract and insulin some growth occurs after 8 days, but there is insignificant incorporation of thymidine at 24 h. It is also of interest that both assays show that keratinocytes are more sensitive to insulin in the absence of the placental extract than in its presence, suggesting that the extract may slightly reduce the requirement for insulin.

Figure 3. Effect of exposure of cells to placental extract on stimulation of thymidine incorporation. Cells were plated in duplicate at 2×10^4 cells per well in 1 ml basal medium plus insulin ($5 \mu\text{g/ml}$). Placental extract ($400 \mu\text{g/ml}$) in 0.1 ml of medium was added to experimental wells after 28 h, (time zero on x-axis) and basal medium alone was added to parallel control wells. One hour before indicated times, $2 \mu\text{Ci}$ of [^3H]thymidine was added to each well, and incorporation was determined after 2 h as described. Points shown are the average $\pm$ range of duplicates. Open circles, + placental extract; solid circles, - placental extract.

To further compare the effect of insulin and placental extract in the two types of assay, we varied the concentrations of placental extract in cultures plated in the presence and absence of insulin. Fig 2 shows that both assays showed a similar dependence on placental extract for increasing cell number (Fig 2a) or thymidine incorporation (Fig 2b) in the absence and presence of insulin. Although the curves do not permit precise determination of half-maximally effective concentrations, the similar concentration dependences in the two assays both in the presence and absence of insulin suggests that the thymidine assay is measuring the growth response of the cultures.

Other Features of Thymidine Incorporation in Keratinocyte Monolayers To determine at what time after exposure to the extract thymidine incorporation increased, we plated keratinocytes in the absence of the extract for 28 h, and then treated the cells with placental extract or control medium. As shown in Fig 3, maximal stimulation occurred after about 17 h of culture, indicating that the stimulation was not the result of a simple stimulation of thymidine transport, which might occur within minutes of addition of the factor, but required prolonged action on the cells. Furthermore, measurement of the TCA-soluble and -insoluble pools (Table I) indicated that although some stimulation of the TCA-soluble pool occurred, a more significant stimulation of the TCA-insoluble or macromolecular pool could be demonstrated. As is shown in the

Table I. Incorporation of Thymidine into TCA-Soluble and -Insoluble Pools

Addition	TCA-Soluble (cpm)	Ratio of Stimulated to Unstimulated Cultures	TCA-Insoluble (cpm)	Ratio of Stimulated to Unstimulated Cultures
None	122	1.0	714	1.0
PX, ^a 200 $\mu\text{g/ml}$	877	7.2	12,782	17.9
PX, 100 $\mu\text{g/ml}$	406	3.3	4,973	7.0
PX _{as} , ^b 200 $\mu\text{g/ml}$	1,340	11.0	18,300	25.6

Keratinocytes were plated with or without additions and incubated with $1 \mu\text{Ci/ml}$ of [^3H]thymidine for 4 h. Cells were then washed twice with phosphate-buffered saline and then extracted sequentially with 0.6 and 2 ml of cold TCA for 10 min. Insoluble residue was solubilized in 0.1 M NaOH containing 1% sodium dodecyl sulfate. TCA-soluble extract was neutralized and both extracts were counted in scintillation fluid.

^aPX, placental extract.

^bPX_{as}, 40% ammonium sulfate precipitate of PX.

table, although the ratio of stimulated to unstimulated radioactivity in the soluble fraction increased in response to the placental extract, the same ratio in the insoluble fraction, representing DNA, was always more than twice that in the soluble fraction, indicating that the increased incorporation into DNA was also due to increased polynucleotide synthesis and was not solely secondary to increased transport.

Because incorporation of radioactivity does not demonstrate what proportion of cells is being stimulated, significant incorporation of radioactivity could result from incorporation by a small and perhaps insignificant fraction of the cells in the culture. To determine the proportion of cells incorporating labeled thymidine in the assay, we labeled the cells for the last 4, 8, or 23 h after addition of placental extract and determined the number of cells with nuclear labeling by autoradiography (Table II). These studies indicated that stimulation of up to 85% of plated cells occurred within 23 h in the presence of the extract, but only 24% of control cells were labeled. If cells were labeled for shorter times near the end of the 23-h labeling period, a higher ratio of stimulated to control cells was obtained. For example, labeling between 19 and 23 h after plating gave a 10:1 ratio of stimulated to control nuclear labeling.

To determine whether the thymidine assay was useful at different times after plating of the cultures and at different cell densities, we plated keratinocytes at three different densities with or without placental extract and compared the incorporation in the cultures. As shown in Fig 4, as cell density increased there was a decrease in the ratio of radioactivity incorporated in stimulated as compared with unstimulated cultures. At higher plating densities (2×10^4 cells/well or higher) the ratio of stimulated to control cultures decreased with increasing time in culture (2 or 3 days), but at the lower density used (10^4 cells/well), the ratio of stimulated to control did not fall, indicating that the assay remained highly sensitive several days after plating of the cultures if cell density did not become too high. The effect of increasing cell density at the time of plating is also illustrated in this experiment; the ratio of stimulated to control incorporation fell at 1, 2, or 3 days of culture as plating density increased. A similar effect of plating at higher densities was demonstrated when the cells were labeled for the last 6 h of a 24-h period (data not shown). Sensitivity of the assay is optimal at about 10^4 cells/well. Therefore, the assay can be performed with good sensitivity at 10^4 cells/well and terminated within 20 h of plating the cells using a 6-h pulse of [3 H]thymidine.

The addition of EGF to plated keratinocytes also increased nuclear labeling (not shown) and thymidine incorporation. As shown in Table III, EGF increased thymidine incorporation in the absence of the placental extract as well as in its presence. Similar results were obtained earlier at this plating density using cell counting to assess growth [5]. Our cultures were grown in the presence of hydrocortisone, which has been shown in previous studies to stimulate their growth [5,9]. Although responsiveness to EGF depends heavily on the presence of hydrocortisone in some types of cells [11], we did not evaluate whether hydrocortisone is required for a response to EGF in keratinocytes because of their poor growth in the absence of hydrocortisone in MCDB 153 medium [5], particularly under the conditions of our assays.

Table II. Autoradiography of Keratinocytes Stimulated by Placental Extract

Duration of Labelling (h)	Percent Labeled Nuclei	
	Stimulated	Control
0-23	84.9	23.5
15-23	76.6	8.3
19-23	49.9	4.8

Keratinocytes were plated in 24-well dishes as described in Materials and Methods and incubated in the presence or absence of 400 μ g placental extract/ml. [3 H]thymidine (1 μ Ci) was added to each well after 0, 15, or 19 h, and the assay was terminated as described after 23 h. Two hundred nuclei were counted in each well, and each experimental point was obtained from duplicate wells.

Figure 4. Effect of duration of culture after plating on thymidine incorporation in stimulated and unstimulated keratinocytes. Keratinocytes were plated in duplicate in basal medium plus insulin (5 μ g/ml) at 1 (circles), 2 (triangles), or 3 (squares) $\times 10^4$ cells per well in 24-well trays in the presence or absence of placental extract (400 μ g/ml). Incorporation was determined as described after 1, 2, or 3 days of culture after a 24-h incubation with 1 μ Ci [3 H]thymidine per well. Numbers in parentheses indicate ratio of radioactivity incorporated in stimulated cultures to that in unstimulated cultures. Solid symbols, + placental extract; open symbols, - placental extract.

If growth factors such as EGF were able to increase cell spreading or cell attachment, wells with added factors would not be comparable to control wells, and increased thymidine incorporation might reflect only an increased number of cells in wells with added growth factor and not an activity increasing proliferative growth. Since spreading was maximal in basal medium after 4 h and did not increase further with added growth factors or matrix proteins (data not shown), cells plated for 6 h or more could not increase their attachment efficiency if addition of the factors was delayed for 6 h after plating. To control for activities that might enhance cell attachment and thereby indirectly increase thymidine incorporation, we plated cells in basal medium without additions for 6 h and then added growth factors and tested for thymidine incorporation (Table IV). These studies showed that placental extract, EGF, and insulin continued to stimulate thymidine incorporation; their effects were therefore not dependent upon their ability to increase the numbers of cells attached to plastic. Plating of cells 6 h before addition of growth factors is a convenient method for assessing growth-promoting activity independent of any activity increasing attachment.

Table III. Effect of EGF and Placental Extract on Thymidine Incorporation in Cultured Keratinocytes

EGF	Placental Extract (μ g/ml)	[3 H]Thymidine Incorporated (cpm) ^a
None	0	1,160 $\pm$ 200
	25	2,040 $\pm$ 120
	200	23,480 $\pm$ 1,420
1 ng/ml	0	11,470 $\pm$ 1,590
	25	16,480 $\pm$ 630
	200	34,470 $\pm$ 910

Keratinocytes were plated as described and assayed for [3 H]thymidine incorporation, as described in Materials and Methods.

^aMean $\pm$ range of duplicate determinations.

Table IV. Effect on Thymidine Incorporation of Placental Extract, Insulin, or EGF Added 6 h after Plating of Cells

Addition	[³ H]Thymidine Incorporated (cpm) ^a
None	123 ± 20
PX, 200 µg	3,038 ± 352
PX, 100 µg	1,738 ± 98
Insulin, 5 µg	807 ± 75
Insulin, 1 µg	443 ± 37
EGF, 5 ng	734 ± 1

Keratinocytes were plated in 24-well trays at 15,000 cells/well in 0.5 ml of basal medium and incubated for 6 h at 37°C. Additions were dissolved and added in 0.5 ml of the same medium. [³H]thymidine (4 µCi) was added to each well after a further 16 h incubation at 37°C, and incorporation was determined after 6 h as described.

^aMean ± range of duplicate determinations.

DISCUSSION

Thymidine incorporation has long been used as an indicator of proliferative growth in skin *in vivo* and in skin organ culture, and it is clear that epidermis can incorporate exogenous thymidine into DNA. In stratified keratinocyte cultures, however, Davison et al [4] found that pulses of labeled thymidine produced high incorporation soon after cells were plated, but then very little incorporation as the cultures became confluent and reached a steady state. Since thymidine incorporation was very high early after plating and then fell after several days while DNA content continued to increase, thymidine incorporation showed no overall correlation with DNA content. Furthermore, assays for thymidine kinase indicated that the cultures may have become less able to phosphorylate thymidine and therefore may have synthesized the nucleotide *de novo*.

Nevertheless, thymidine incorporation has been used by others in keratinocytes *in vitro*, for example by Milstone and LaVigne [12] to assess promotion of growth; they found that it correlated well with growth promotion as assessed by cell counting. It is possible that the peak in incorporation demonstrated early after plating by Davison et al and the subsequent fall they reported were measurements of the rate of DNA synthesis; therefore, although the DNA synthetic rate does not correlate with DNA content, the synthetic rate may correlate, as suggested by our data, with stimulation of growth. After several days of culture, the DNA-synthetic rate, and therefore the rate of thymidine incorporation under pulse-labeling conditions, may fall even as the DNA content of the cultures continues to increase. In addition, the relationship of growth and thymidine incorporation may hold better in 0.1 mM Ca²⁺, in which cells are maintained in a monolayer, than in a stratified culture.

Our studies used labeled thymidine to assess the difference in DNA synthesis between nonproliferating and stimulated cultures in the first several days after plating. These cultures were maintained as a monolayer by reduction of Ca²⁺ concentration in the medium to 0.1 mM, which prevents stratification [13]. Under these conditions, plated keratinocytes grow rapidly in properly supplemented medium. Comparison of the dependency on insulin and placental extract indicates that a thymidine incorporation assay gives results very similar to those from direct assay of cellular proliferation, since stimulation can be demonstrated in both assays, and such stimulation shows similar concentration dependence for both factors. If the cells are grown at higher densities than those described here, however, the requirement for insulin, and perhaps other growth factors, is decreased (data not shown). It is likely that the need for insulin would appear to be reduced if even higher densities were used in the thymidine assay.

The peak in thymidine incorporation occurs after a lag period of about 15 h, and coincides with the onset of nuclear labeling, as demonstrated by autoradiography of labeled nuclei. The degree of stimulation and time of maximal labeling are similar in both nuclear labeling and thymidine incorporation if both studies are performed under similar conditions, further suggesting that similar phenomena are being measured.

Substantial interassay variation in [³H]thymidine incorporation can be observed in our data, and is probably determined by the condition of the cells at the time of plating. For example, in the presence of 400 µg placental extract/ml, and in the absence of insulin, [³H]thymidine incorporation varied from 1.2 × 10⁶ dpm (Fig 1b) to 5 × 10⁵ dpm (Fig 2b). Even more variation is apparent in comparison of cells with no placental extract and insulin at 5 µg/ml in Fig 1b with cells under similar conditions (10⁴ cells/well) in Fig 4. Presumably because keratinocytes begin to terminally differentiate when they become confluent, even when cultured at 0.1 mM Ca²⁺, the condition of the culture at the time of trypsinization for use in the assay has a major influence on plating efficiency and therefore on subsequent responsiveness in the assay, and this problem is reflected in the major quantitative variations in the results. Also, minor alterations in the exposure to trypsin have a major effect on subsequent plating efficiency. Finally, keratinocytes become less responsive to growth factors at higher passages. For these reasons, results cannot be compared between assays; comparisons between different conditions and growth factors must be made in the same experiment on the same batch of cells. Interassay variation can be reduced by standardization of conditions of trypsinization and plating, and by use of cells at similar degrees of confluency in all assays, but the variations cannot be removed altogether.

Under the conditions of this assay we have demonstrated substantial stimulation of thymidine incorporation by EGF, which has long been known to stimulate growth of keratinocytes in culture [5,7,9], although significant stimulation of thymidine incorporation in stratified cultures of keratinocytes has not been demonstrated in response to EGF. The assay facilitates rapid study of growth-stimulating fractions, and has been useful for partial purification of a keratinocyte-stimulating activity in extracts of human placenta. Because the assay can be adjusted to produce negligible incorporation of radioactivity in the absence of the factor to be assayed, extremely high ratios of experimental to control samples can be obtained. For example, in the absence of EGF and the placental extract, thymidine incorporation is negligible, and addition of the extract permits as much as 50-fold stimulation, whereas in the presence of EGF, about 3-fold stimulation is commonly produced by the placental extract over that in the presence of EGF alone. The assay can also be used to assess the duration of G₁ and the rate of entry into S [14].

We thank Richard E. Payne, Jr., for excellent technical assistance, and P. Schwartz and L. Milstone for helpful discussions.

REFERENCES

- Weinstein GD, McCullough JL, Ross P: Cell proliferation in normal epidermis. *J Invest Dermatol* 82:623–628, 1984
- Bickenbach JR, Mackenzie IC: Identification and localization of label-retaining cells in hamster epithelia. *J Invest Dermatol* 82:618–622, 1984
- Milstone LM and LaVigne JF: Heterogeneity of basal keratinocytes: nonrandom distribution of thymidine-labeled basal cells is not a technical artifact. *J Invest Dermatol* 84:504–507, 1985
- Davison P, Liu S, Karasek M: Limitations in the use of [³H]thymidine incorporation into DNA as an indicator of epidermal keratinocyte proliferation *in vitro*. *Cell Tissue Kinet* 12:605–614, 1979
- O'Keefe EJ, Payne RE Jr, Russell N: Keratinocyte growth-promoting activity from human placenta. *J Cell Physiol* 124:439–445, 1985
- O'Keefe E, Battin T, Payne RE Jr: Epidermal growth factor receptor in human epidermal cells: direct demonstration in cultured cells. *J Invest Dermatol* 78:482–487, 1982
- Rheinwald JG, Green H: Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 6:331–334, 1975
- McKeehan WL, McKeehan KA, Hammond SL, Ham RG: Improved medium for clonal growth of human diploid fibroblasts at low concentrations of serum protein. *In Vitro* 13:399–416, 1977

9. Boyce ST, Ham RG: Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *J Invest Dermatol* 81:33s-40s, 1983
10. Savage CR Jr, Cohen S: Epidermal growth factor and a new derivative. *J Biol Chem* 247:7609-7611, 1972
11. Hosokawa M, Philips PD, Cristofalo VJ: The effect of dexamethasone on epidermal growth factor binding and stimulation of proliferation in young and senescent WI38 cells. *Exp Cell Res* 164:408-414, 1986
12. Milstone LM, LaVigne JF: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin induces hyperplasia in confluent cultures of human keratinocytes. *J Invest Dermatol* 82:532-534, 1984
13. Hennings H, Michael D, Cheng C, Steinert P, Holbrook K, Yuspa SH: Calcium regulation of growth and differentiation in mouse epidermal cells in culture. *Cell* 19:245-254, 1980
14. O'Keefe EJ, Pledger WJ: A model of cell cycle control: sequential events regulated by growth factors. *Mol Cell Endocrinol* 31:167-186, 1983

CERTIFYING EXAMINATION OF THE AMERICAN BOARD OF DERMATOLOGY

October 30, 31, 1988

In 1988, the Certifying Examination of the American Board of Dermatology will be held at the Holiday Inn O'Hare Airport in Rosemont, Illinois on October 30 and 31, 1988. The deadline for receipt of applications is May 1, 1988.

The Dermatopathology Special Qualification Examination will be held in Dallas, Texas on November 12, 1988. The deadline for receipt of applications is July 1, 1988.

The next examination for Special Qualification in Dermatological Immunology/Diagnostic and Laboratory Immunology will be held on November 7, 1989. The deadline for receipt of applications will be April 1, 1989.

For further information on these examinations, please contact: Clarence S. Livingood, M.D., Executive Director, AMERICAN BOARD OF DERMATOLOGY, Henry Ford Hospital, Detroit, MI 48202.