We have demonstrated for what we believe to be the first time in a carefully controlled experiment that moderate levels of realistic noise, presented at appropriate times throughout the day, can produce sustained elevations in blood pressure without producing significant changes in auditory sensitivity. While extrapolation from one species to another must always be undertaken with caution, we have provided evidence, based on a primate model, that these two categories of event may occur independently in humans exposed to moderately intense noise over long periods of time. Further, we have demonstrated that noise effects do not necessarily dissipate when the noise ends.

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6. The A-weighting attenuates response of a sound level meter or other measuring device below 1000 Hz and above 4000 Hz. The frequency response thus approximates the curve of human audibility near threshold levels. The A-weighting is a widely accepted predictor of human reaction to noise.
7. ABR is the acronym recommended by the joint U.S.A.-Japan Seminar on Auditory Responses from the Brainstem, Honolulu, Hawaii, 27 November to 1 December 1979 [See H. Davis, *Laryngoscope* **89**, 1336 (1979)]. The latency of the ABR component has proved to be the most consistent and reliable diagnostic index for subcortical auditory pathway function.
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# An Endocrine Approach to the Control of Epidermal Growth: Serum-Free Cultivation of Human Keratinocytes

Abstract. *Human keratinocytes, derived from the skin of newborns and of adults, were grown in the complete absence of serum, in a hormone-supplemented medium on fibronectin-coated cell culture dishes at low seed density. The cell culture medium consisted of Medium 199 containing epidermal growth factor, triiodothyronine, hydrocortisone, Cohn fraction IV, insulin, transferrin, bovine brain extract, and trace elements. Removal of the brain extract from the hormone supplement had a greater negative impact on proliferation of the keratinocyte cultures than did the removal of epidermal growth factor, hydrocortisone, and triiodothyronine or Cohn fraction IV. The growth of keratinocytes in this hormone-supplemented medium suggests that control of keratinocyte growth depends in large part on endocrine stimulation by other body organs, including the brain.*

The serum dependence of mammalian cell growth in vitro is mediated by combinations of serum-derived hormones and growth factors (*1*). Certain fastidious cells incapable of growth in serum-supplemented medium require the use of feeder layers, cell-conditioned medium, very high seed densities, or organ extracts (*2*, *3*) as possible sources of additional specific growth factors. Human keratinocytes have been successfully cultivated on lethally irradiated 3T3 cell feeder layers in the presence of serum (*4*), with cell growth being further improved by the addition of epidermal growth factor and hydrocortisone to the medium (*5*).

We recently demonstrated that a fibronectin matrix provides a biologically relevant surface for the attachment of human keratinocytes in vitro (*6*). The use of a fibronectin-coated surface permits keratinocyte growth at low seed density in the absence of a 3T3 cell feeder layer or other contaminating cell types (*6*), and at serum concentrations considerably below those apparently needed in other systems (*4–7*). This approach has enabled us to identify factors necessary for the serum-free cultivation of human keratinocytes and has resulted in an appreciation of the brain as an important source of keratinocyte growth-promoting activity.

Primary cultures of human keratinocytes were obtained from trypsin-split epidermal sheets of human skin specimens as previously described (*5*). Both neonatal foreskins and facial skin from adults aged 56 to 62 years were used in the experiments reported here. Single-cell suspensions were plated on cell culture dishes previously coated with purified human fibronectin (10 μg/cm$^2$) (*7*) at

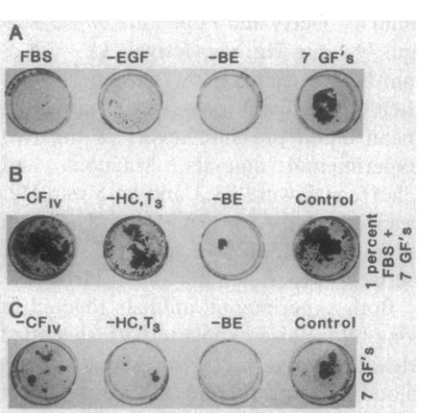


Fig. 1. Human keratinocyte growth in a serum-free environment with and without the addition of 1 percent fetal bovine serum: A single cell suspension of human keratinocytes was plated at a seed density of 5 × 10$^4$ cells per square centimeter on cell culture dishes (35 mm, Falcon) coated with 10 μg of human fibronectin per square centimeter. The medium was replaced every 2 to 3 days with the appropriate medium. The hormone supplement (*7 GF's*) contained 10 ng of epidermal growth factor per milliliter (*EGF*, Collaborative Research, Waltham, Mass.), 10 μg of insulin per milliliter (*I*, Sigma), 10 μg of transferrin per milliliter (*T*, Sigma), 5 × 10$^{-5}$M hydrocortisone (*HC*, Sigma), 10$^{-9}$*M* triiodothyronine ($T_3$, Sigma), 150 μg per milliliter of human Cohn fraction IV (*CF$_{IV}$*, Armour Pharmaceuticals, Chicago), 150 μg per milliliter of bovine brain extract (*BE*) and trace elements in Medium 199 (Gibco, Grand Island, New York). (A) (Left to right) 20 percent FBS control; seven growth factors minus EGF (−*EGF*); seven growth factors minus bovine brain extract (−*BE*); and hormone supplement control (*7 GF's*). (B) Seven growth factors plus 1 percent FBS minus human Cohn fraction IV (−*CF$_{IV}$*); seven growth factors plus 1 percent FBS minus triiodothyronine and hydrocortisone (−*HC*, $T_3$); seven growth factors plus 1 percent FBS minus bovine brain extract (−*BE*); seven growth factors plus 1 percent FBS control. (C) Seven growth factors minus human Cohn fraction IV (−*CF$_{IV}$*); seven growth factors minus triiodothyronine and hydrocortisone (−*HC*, $T_3$); seven growth factors minus bovine brain extract (−*BE*); and the hormone supplement control (*7 GF's*). After 15 days of growth, the cultures were fixed with 4 percent Formalin for 40 minutes and stained with 1 percent Rhodanile blue (MCB, Norwood, Ohio) for 1 hour.


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a cell density of $5 \times 10^4$ cells per square centimeter. Cultures supplemented with 20 percent fetal bovine serum (FBS) in Medium 199 served as controls. Experimental cultures were supplemented with a hormone "cocktail" in the presence and absence of 1 percent FBS. The hormone supplement contained epidermal growth factor (10 ng/ml), insulin (10 μg/ml), transferrin (10 μg/ml), hydrocortisone ($5 \times 10^{-5} M$), triiodothyronine ($10^{-9} M$), human Cohn fraction IV (150 μg/ml), bovine brain extract (150 μg/ml), and trace elements (*8*) in Medium 199. Insulin and transferrin are standard components of serum-free media designed for the growth of established mammalian cell lines in vitro (*1, 9*); epidermal growth factor and hydrocortisone are established mitogens for human keratinocytes (*5*). Triiodothyronine is a hormone that has proved useful in the cultivation of neural-derived cells (*10*) which, like keratinocytes, are of ectodermal origin. The Cohn fraction IV preparation, a potent source of the insulin-like growth factors (*11*), was prepared by extraction in 0.05*N* acetic acid for 2 hours followed by gentle boiling for 30 minutes. The extract was clarified by centrifugation and the supernatant neutralized with 1*N* NaOH. The supernatant was dialyzed against 10 m*M* sodium phosphate buffer, *p*H 7.0, containing 15 m*M* NaCl and stored as a lyophilized powder. The bovine brain extract, a source of an endothelial cell growth factor (*3*) and fibroblast growth factor (*2, 12*), was prepared as previously described (*3*).

The keratinocyte cultures were grown in the appropriate supplements for 15 days. At this time the plates were fixed and stained (Fig. 1). Keratinocytes were identified by their ability to stain red-purple with Rhodanile blue and by their characteristic morphology consisting of tightly apposed polygonal cells in the center of the colonies (Fig. 2A) (*4*). Proliferation was most active at the periphery of the keratinocyte colonies where larger, more loosely aggregated cells were apparent (Fig. 2B). Stratification was evident in most of the keratinocyte colonies. Scattered fusiform cells, presumably representing either melanocytes or fibroblasts, were visible near the colony edge. These cells were also present in epidermal skirts surrounding human skin explants grown in the hormone supplemented, serum-free medium (Fig. 2C). Although the fibroblast-like cells do not propagate under these conditions, it is possible that they serve as helper cells and provide additional growth-promoting factors. The absence of fibroblast proliferation and overgrowth of the keratinocyte cultures grown in the hormone-supplemented medium can be attributed to the lack of platelet-derived growth factor, a potent and necessary fibroblast mitogen (*13*), in the hormone supplement. The presence of a positive stain with Rhodanile blue, indicating the presence of keratin proteins, suggests that the keratinocytes remain capable of differentiation in the hormone-supplemented environment.

Although the cultures grown in the serum-free, hormone-supplemented medium contained large and fused keratinocyte colonies, easily in excess of 500 cells, the level of keratinocyte growth was enhanced by the addition of 1 percent FBS to the hormone supplement (Fig. 1). These results suggest that although this hormone supplement does permit the growth of keratinocytes, additional serum-derived components are required for optimum cell growth.

Removal of either the brain extract or epidermal growth factor from the hormone-supplemented medium resulted in a dramatic reduction in keratinocyte growth in the hormone control as well as the 1 percent FBS supplemented cultures (Fig. 1). In contrast, the absence of either the Cohn fraction IV preparation or triiodothyronine and hydrocortisone from the hormone supplement resulted in only a slightly diminished growth response. The human keratinocyte mitogen present in the brain extract appears to be a high-molecular-weight protein with an acid isoelectric point (*3*). These results suggest that neural tissue contains one or more mitogenic factors important for keratinocyte growth and, conversely, that keratinocyte growth depends in large part on endocrine stimulation by other body organs, including the brain.

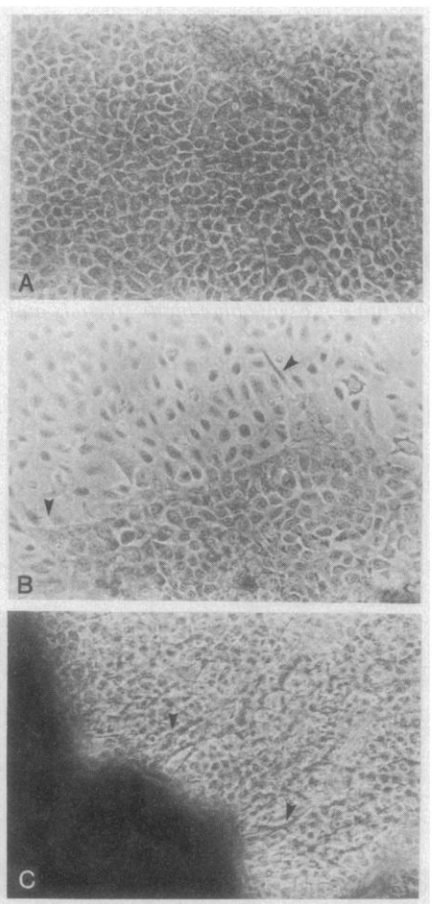


Fig. 2. Phase contrast photomicrographs of human keratinocytes grown in a serum-free environment. Human keratinocytes were grown from single-cell suspensions at seed densities of $2 \times 10^4$ to $5 \times 10^4$ cells per square centimeter, and from explant fragments of human skin on cell culture dishes coated with 10 μg of human fibronectin per square centimeter (*5*), in Medium 199 in the presence of the hormone supplement. (A) Tightly packed human keratinocytes at the center of a large colony. (B) Larger, loosely packed keratinocytes at the periphery of a colony with fibroblast-like cells (arrows). (C) Human keratinocyte outgrowth from an explant with residual fibroblast-like cells (arrows). (Magnification ×100.)

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was centrifuged (13,800*g*, 30 minutes) and the supernatant stored as a lyophilized powder. The extract was reconstituted with 50 m*M* tris-HCl, *p*H 7.4, containing 150 m*M* NaCl and 0.5 m*M* EDTA and applied to a Sephadex G-100 column equilibrated in the reconstitution buffer. The keratinocyte growth-promoting activity chromatographed with a $K_a$ of approximately 0.3. Preparative isoelectric focusing (Bio-Rad Labs) between *p*H 10 and *p*H 3 was also performed with the brain extract. The extract was dialyzed (5000 molecular weight cutoff) against 100 μ*M* tris-HCl, *p*H 7.0, for 18 hours prior to isoelectric focusing. The keratinocyte growth-promoting activity migrated to an isoelectric point between *p*H 4 and *p*H 5.

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## Estrogen Receptors in the Nidatory Sites of the Rat Endometrium

Abstract. *In ovariectomized rats treated with progesterone, implantation was induced by a minute dose of 17β-estradiol. Twenty-four hours later, the concentrations of estradiol receptor in nuclear and cytosol fractions prepared from the endometrium surrounding the blastocyst and the inter-implantation areas remained very low. This indicates that estrogen was not secreted by the blastocyst. The higher receptor content in cytosol from inter-implantation sites may reflect modifications accompanying the decidual reaction since our results show that there is no translocation of the receptor to the nuclei. The choice of the dye used to reveal the implantation sites is critical, since Trypan blue but not Evans blue binds steroids and thereby interferes with receptor measurements.*

For ovoimplantation to be initiated, egg development and preparation of the endometrium must be closely synchronized. In the rat, the timing of endometrial preparation depends on a precise sequence of hormonal reactions. Thus only after the endometrium has been primed with progesterone for at least 48 hours can it be induced by estradiol to become receptive to the blastocyst 18 hours later (*1*). Although progesterone priming may be essential in all species, estradiol intervention does not appear to be a common process (*2–4*). It has been hypothesized that, at the time of implantation, the blastocyst itself influences endometrial function locally by releasing estradiol (*5–6*). Synthesis of estradiol by the blastocyst has been described in some species, and it has been suggested in other species that the egg accumulates estradiol in the ovary and releases it later in the uterus (*6–7*).

In the rat there is no direct evidence that estrogens are synthesized or released by the blastocyst. Furthermore, in rats that are ovariectomized before the evening of day 4 of pregnancy and then treated daily with progesterone, ovoimplantation is delayed until a minute amount of estradiol is administered intravenously or locally (*1*). If, indeed, the rat embryo released estradiol locally, this should induce translocation of the estradiol receptors from the cytoplasm to the nucleus in the adjacent endometrial cells.

In the study described here, pregnant rats were ovariectomized under a dissecting microscope (*8*) on day 3 of pregnancy (appearance of vaginal sperm was designated day 1 of pregnancy) and were then treated daily with 4 mg of progesterone; thus any circulating estrogens of maternal origin were removed. Implantation was induced on day 8 postcoitum by a single injection of estradiol (0.25 μg per rat) and the animals were killed 24 hours later. The implantation sites were revealed by injecting intravenously 1 ml of 0.5 percent Evans blue via the femoral vein 20 to 30 minutes before the rats were killed (*9*). The uteri were removed and the blue (implantation sites) and nonblue (inter-implantation) areas of the endometrium were collected and assayed for estradiol receptor. Previous studies in our laboratory had shown that the concentration of estrogen receptor in the myometrium is not modified during implantation (*10*). Control animals, in which the oviducts containing fertilized eggs were removed during ovariectomy, were treated in a similar way.

The concentrations of estradiol receptor in cytosols prepared from uteri of the control animals and from the inter-implantation (nonblue) areas of the uteri from the pregnant animals were similar (Table 1), whereas estradiol receptor in cytosols from implantation (blue) areas was significantly decreased ($P < .001$, *t*-test). This decrease amounts to 53 percent when the receptor content is expressed per microgram of DNA and 63 percent when expressed per milligram of protein. However, in both the implanta-

Table 1. Estradiol receptors in the implantation (blue area) and inter-implantation (nonblue area) sites. Wistar rats were prepared as described in the text; the uteri were removed, slit longitudinally, and placed at 0°C. All other operations were carried out at 0°C unless otherwise specified. The blue and nonblue regions of the endometrium were collected by gentle scraping and homogenized in buffer (250 m*M* sucrose, 3 m*M* $MgCl_2$, 10 m*M* tris-HCl, *p*H 7.4). Cytosolic and nuclear fractions were prepared by centrifugation (700*g* for 10 minutes). The cytosol receptor was assessed by the exchange technique as described by Katzenellenbogen *et al.* (*18*), slightly modified (*19*), and protein determinations were carried out according to Lowry (*20*). The nuclei were washed in a 0.5 percent Triton-X 100 solution in buffer, and washed twice in buffer, then assayed by the [$^3$H]estradiol exchange method as described by Clark *et al.* (*13*). At the end of the incubation period, the nuclei after being washed twice were subjected to acid hydrolysis; one half of the hydrolysate was counted for radioactivity, the other was used for DNA determination (*21*).

| Fraction | Uteri from control rats | | Uteri from rats injected with Evans blue | | | |
|---|---|---|---|---|---|---|
| | | | Inter-implantation sites | | Implantation sites | |
| | Per microgram of DNA (fmole) | Per milligram of protein ($10^{-13}$ mole) | Per microgram of DNA (fmole) | Per milligram of protein ($10^{-13}$ mole) | Per microgram of DNA (fmole) | Per milligram of protein ($10^{-13}$ mole) |
| Cytosol | 7.31 ± 0.52 | 4.45 ± 0.31 | 7.25 ± 0.61 | 4.11 ± 0.70 | 3.40 ± 0.43 | 1.50 ± 0.38 |
| Nuclear | 0.060 ± 0.035 | | 0.056 ± 0.099 | | 0.032 ± 0.011 | |


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