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Absence of tumorigenicity in athymic mice by normal human epidermal keratinocytes after culture in serum-free medium

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Summary

The very rapid growth rate (1 population doubling/day) of normal human epidermal keratinocytes (HK) cultured in serum-free medium can be utilized for wound closure in burn treatment. However, rapid growth in vitro may present the possibility of neoplastic transformation. To investigate this possibility, HK were cultured from primary isolation to large populations in MCDB 153 medium supplemented with epidermal growth factor (EGF, 10 ng/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), and Bovine Pituitary Extract (BPE, 70 μg/ml). HK were studied for their ability to form tumors in athymic mice after subcutaneous inoculation. Sixteen separate HK strains were inoculated from primary cultures, or from secondary cultures either before or after storage in liquid nitrogen. Transformed cell lines, SCC 13 and FL, derived from human epithelial carcinomata were used as controls for tumor formation. HK formed no tumors (0/79) after 26 weeks incubation, SCC 13 formed nodular tumors (3/5) after 20 weeks incubation, and FL formed tumors (5/5) after 4 weeks incubation. HK cells were not found by histological examination of inoculation sites of keratinocyte cultures derived from primary culture from skin. In contrast, palpable tumors from both SCC 13 and FL were returned to tissue culture and continued to proliferate. These results support the conclusion that the rapid growth rate of human epidermal keratinocytes in vitro can be attributed to permissive culture conditions, and not to neoplastic transformation.

Keywords: tumorigenicity; cultured keratinocytes; serum-free medium; cultured skin grafts; burns; skin carcinoma

Introduction

Cultured human epidermal keratinocytes (HK) have been utilized as a substitute for split-thickness skin graft in the treatment of skin wounds, including burns [1,10,13,15,18]. The very rapid growth rate (1 population doubling/day) [2] of HK allows large expansions of area coverage (>1000-fold) by epidermal cell sheets in relatively short periods of time (3 weeks) [3,4,14]. Although HK cultures become growth arrested in the absence of exogenous mitogenic agents [19,23], their rapid growth rate may result from, or contribute to, neoplastic changes in the cellular population. If neoplastic transformation were to occur in the cultured epithelium and it were

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transplanted for purposes of wound closure, cells in the healed skin would have increased probability of developing into squamous cell or basal cell carcinomata.

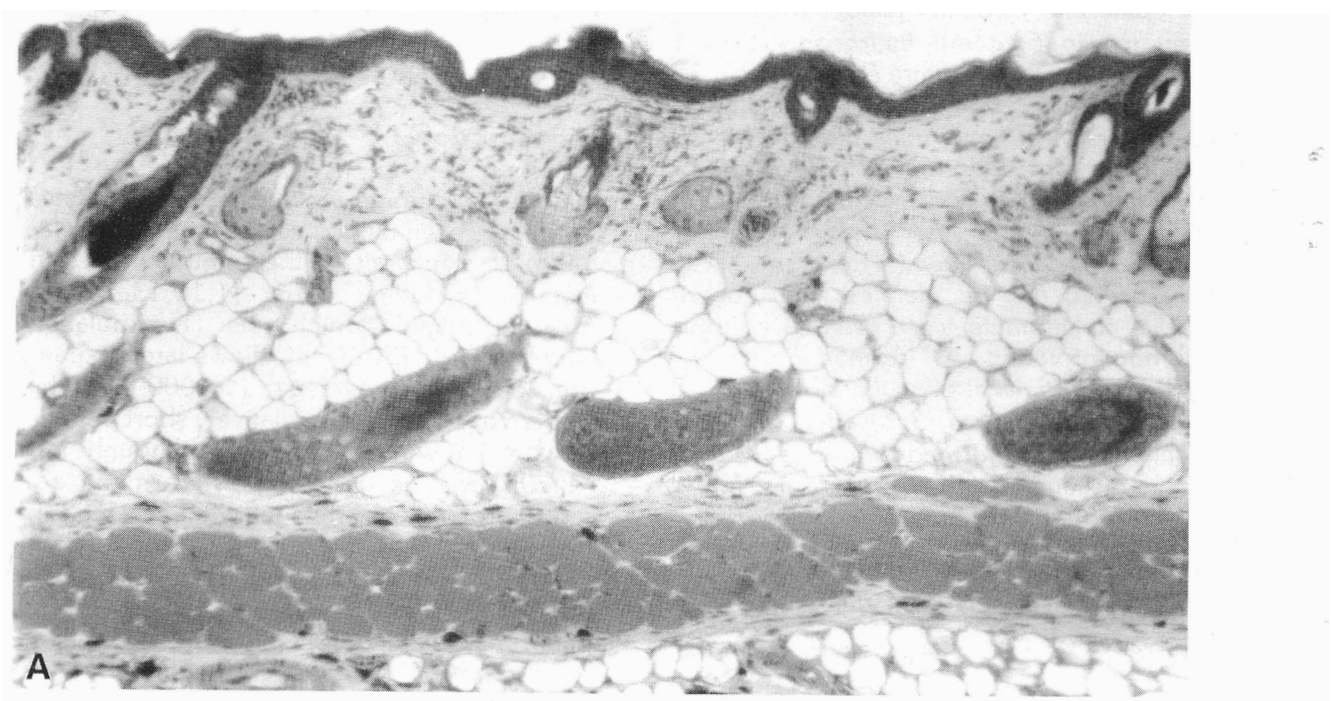
Characters of normal cells in culture include finite proliferative lifespan [2,16], dependence on surface attachment for growth, and growth inhibition after confluency [22]. In addition, normal HK differentiate in response to elevated calcium concentration in the culture medium [2,17]. Transformation of cultured cells can cause the loss of one or more of these normal characters, and can result in an 'immortal' lifespan, growth independent of substrate attachment, and/or continued cell growth after confluency. Malignant transformation of HK is also accompanied by defective terminal differentation [20].

Tumorigenicity in athymic mice is a widely accepted index of malignant transformation in vivo [12]. Recognizing that cellular growth is influenced by the site of implantation, subcutaneous inoculation is considered the most appropriate site to test growth of human keratinocytes from in vitro cultures as a model for skin tranplantation. To evaluate the potential for HK transformation prior to transplantation for wound closure, suspensions of HK cells from serum-free cultures were inoculated subcutaneously into athymic mice and studied for 26 weeks. No tumor formation was detected externally, or by histologic examination of the injection site. Comparable experimental methods using Fogh-Lund (FL) or squamous cell carcinoma (SCC) 13 cells produced palpable nodular tumors in 4 or 20 weeks, respectively. These findings support the conclusion that transplantation of cultured human epidermal keratinocytes for wound closure presents no detectable risk of malignant carcinoma to the patient.

Materials and Methods

Cells studied

Three different types of cultured human



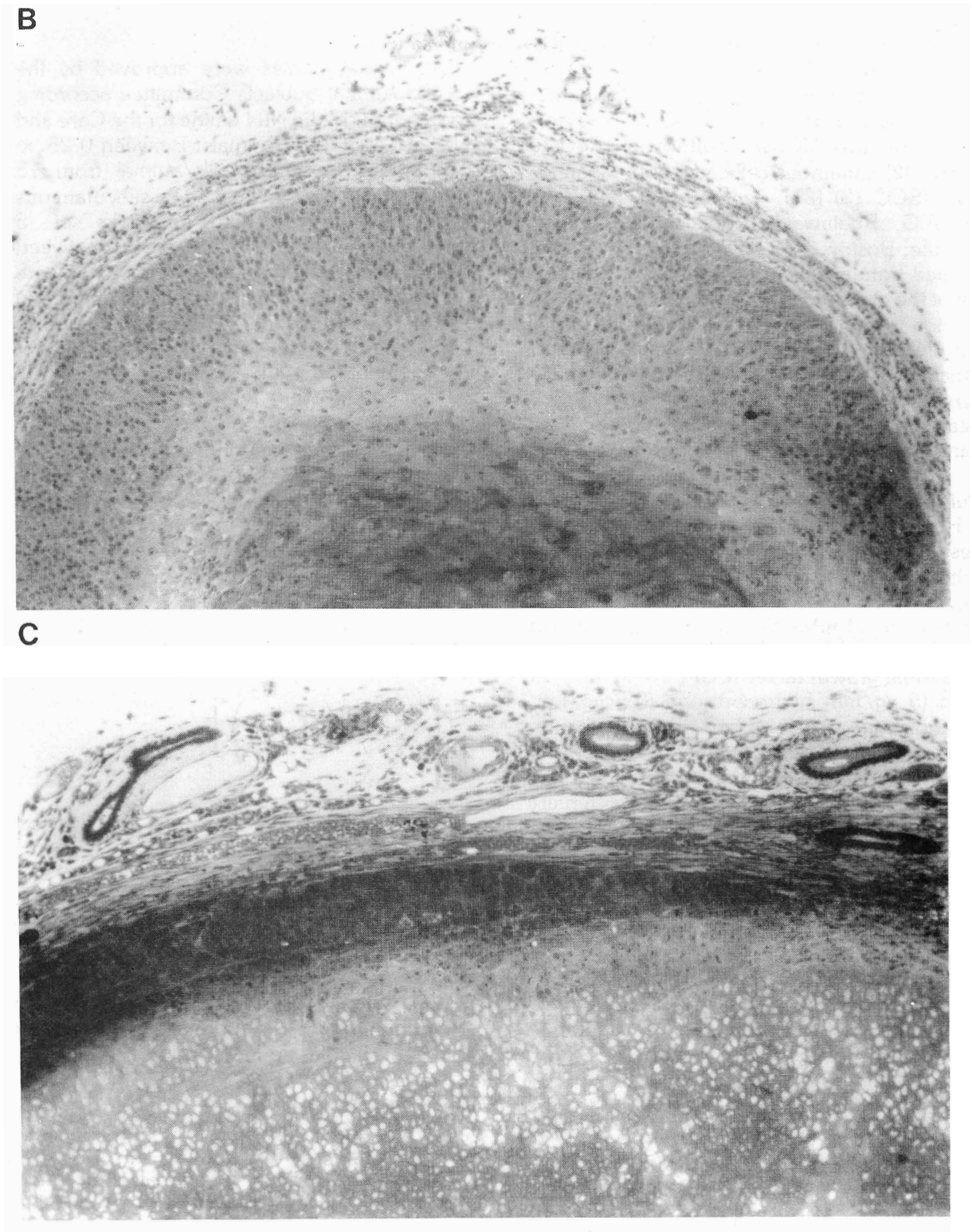


Fig. 1. Histology of inoculation sites. (A) HK site, normal skin morphology with no tumor formation; (B) SCC13 nodular tumor with epithelial morphology; (C) FL nodular tumor with epithelial morphology and necrotic center.

epithelial cells were included in the study: (1) human epidermal keratinocytes (HK) which were derived from skin tissue of apparently healthy donors that was obtained with approval of the UCSD Institutional Review Board; (2) squamous cell carcinoma keratinocytes (SCC 13) [21], generously provided by Dr. J.G. Rheinwald (Dana Farber Cancer Institute; Boston, MA). SCC 13 was originally isolated from a clinical squamous cell carcinoma of the oral mucosa; (3) transformed human amnion keratinocytes (FL) [11] obtained from the American Type Culture Collection (Rockville, MD 20852). FL was originally derived from normal human amnion tissue, established into culture in 1956, and became transformed spontaneously soon thereafter.

Culture conditions

HK were isolated from neonatal foreskin, fresh cadaver skin or surgical discard, and cultured in serum free 'Molecular, Cellular and Developmental Biology' medium (MCDB 153) containing 0.1 mM calcium and supplemented with bovine pituitary extract (BPE, 70 μg/ml), epidermal growth factor (EGF, 10 ng/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), ethanolamine (0.1 mM) and phosphoethanolamine (0.1 mM) [2–4]. HK strains were inoculated as either secondary cultures that had not been previously stored frozen, or as tertiary cultures after recovery from frozen storage. Medium for frozen storage contained 10% v/v dimethylsulfoxide (DMSO).

SCC 13 were grown with 3T3 feeder layers for stock cultures in MCDB 153 medium supplemented with 20% fetal bovine serum (FBS). For preparation of inocula, SCC 13 were cultured in serum-free MCDB 153 medium containing 0.3 mM calcium and supplemented as above, but without EGF [21]. FL were obtained from the American Type Culture Collection (ATCC, CCL 62) and grown in stock culture in Dulbecco's Modified Eagle's Medium plus 10% FBS. For inocula, FL were grown in MCDB 153 medium plus 10% FBS.

Inoculation

All animal studies were approved by the UCSD Animal Subjects Committee according to standards of the NIH Guide for the Care and Use of Laboratory Animals. Between 0.25×10^7 and 1.0×10^7 cells/animal from 16 strains of HK were inoculated by subcutaneous injection into the left foreflanks of 5 animals/strain. For SCC 13 and FL, between 0.5×10^7 and 1.0×10^7 cells/animal were inoculated as above into 5 animals/line. Any tumors were removed at the time of sacrifice and replaced into their respective tissue culture conditions.

Tumor measurement

Each week following inoculation, the external size of any tumor was measured along its shortest (width, 'W') and longest (length, 'L') axes. An estimate of tumor volume was calculated from the measurements, as the volume of a cylinder with hemispherical ends, by the equation:

$$\text{Calculated volume (ml)} = \left[\pi\left(\frac{W}{2}\right)^2\left(L - W\right)\right] + \left[\frac{4}{3}\pi\left(\frac{W}{2}\right)^3\right]$$

Final tumor volume was determined directly by fluid displacement of the resected tumor at the time of sacrifice of each group of animals. Histology and electron microscopy were performed by standard techniques to identify epithelial cell type of tumors by desmosome ultrastructure.

Results

External appearance

No tumors formed in animals inoculated with any of 16 strains of HK cells, but palpable

nodular tumors formed in animals inoculated with SCC 13, and FL.

Histology

Figure 1 shows representative histologies from the inoculation sites of animals receiving HK (Fig. 1A), SCC 13 (Fig. 1B) or FL cells (Fig. 1C). No abnormal histological features were found in animals injected with HK cells. The epithelial cell type of the tumors formed from SCC 13 and FL is demonstrated by light microscopy. In addition, transmission electron microscopy of both SCC 13 and FL tumors identified desmosomes between cells confirming the epithelial phenotype of the tumors (data not shown).

Tumor progression

Figure 2 shows the increase of calculated tumor volume versus time in weeks. With HK, small nodules which represented the original inocula were present during the first 1–2 weeks, but receded and resolved (N = 79; tumor frequency = 0/79). With SCC 13, small nodules formed that did not recede, but grew slowly during the first 3 months before proceeding into more rapid growth in the fourth and fifth months (N = 5; tumor frequency = 3/5). With FL, the initial inoculum formed rapidly growing tumors that required sacrifice of the animal after 4 weeks of incubation (N = 5; tumor frequency = 5/5).

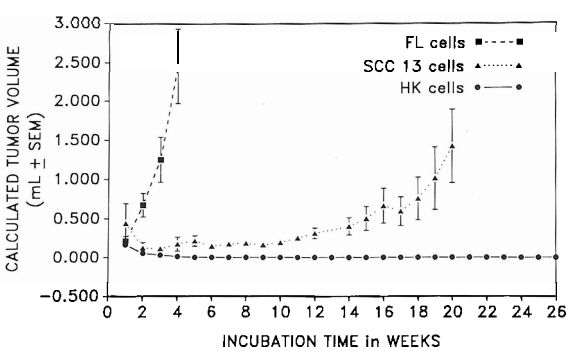


Fig. 2. Tumor progression vs time. Tumor size (ml ± S.E.M.) was calculated as described in Materials and Methods. HK (circles) showed small nodules which regressed and resolved after 2 weeks. SCC 13 (triangles) grew slowly during the first 12 weeks and increased in size more rapidly between 14 and 20 weeks incubation. FL (squares) grew so aggressively that animals were sacrificed after 4 weeks incubation.

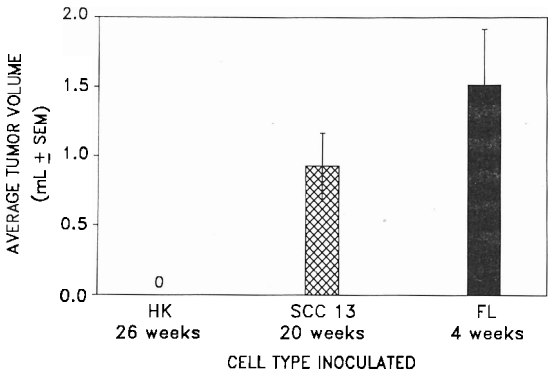


Fig. 3. Final tumor volume. Average tumor volume (ml ± S.E.M.) was determined directly by fluid displacement at the time of sacrifice. Left, HK = 0 ± 0. Center, SCC 13 = 0.93 ± 0.24. Right, FL = 1.52 ± 0.40.

Final tumor volume and tissue culture

Figure 3 shows the average tumor volume as determined by fluid displacement after resection of tumors at the times of sacrifice. The average volume (ml + S.E.M.) for HK = 0 ± 0 after 26 weeks, for SCC 13 = 0.93 ± 0.24 after 20 weeks, and for FL = 1.52 ± 0.40 after 4 weeks of incubation. Both SCC 13 and FL were successfully re-established into tissue culture after resection and disaggregation of the tumors (data not shown).

Discussion

Increased mitotic rate and migration of epidermal keratinocytes are characteristics of normal healing of skin wounds [7]. After re-epithelialization is complete, keratinocyte growth rate slows to re-establish the steady state equilibrium between proliferating and differentiating keratinocytes of undamaged epidermis. The proliferative rate of non-trans-

formed HK is regulated by the normal processes of epidermal differentiation and wound healing, both of which operate predominantly by the presence or absence of exogenous factors. Rapid growth of cultured HK is interpreted as the expression of a normal character of wound healing physiology that is promoted by permissive culture conditions, and by absence of inhibitory factors of regulation.

By comparison, neoplastic epidermal keratinocytes are defective in differentiation, and may also exhibit a more rapid mitotic rate than basal keratinocytes in normal epidermis. The failure to respond to exogenous regulatory factors distinguishes transformed HK from their normal counterparts, and identifies transformation as an endogenous event that is most often an irreversible alteration of gene regulation and expression at the molecular level. The type (chemical, viral, physical) and degree of transformation defines whether unregulated growth is benign and localized, or sufficient to induce the malignant characteristics of invasion and metastasis.

The absence of tumor formation by 16 strains of cultured human epidermal keratinocytes (HK) provides strong evidence that rapid proliferation of these cells in serum-free culture medium does not induce endogenous changes that result in malignant transformation. Histologic examination of inoculation sites in animals receiving cultured HK showed no evidence of epithelial cells at or near the site. The resorption of the cultured HK demonstrates either that no endogenous changes to the cells occurred in vitro, or that any endogenous change that may have occurred was not sufficient to allow HK proliferation to escape the regulatory environment of the host.

Although no tumors were detected in this study, it is possible that tumors may eventually form if greater numbers of HK strains are tested, or if other anatomic sites are inoculated. However, after observation of greater than 100 HK strains cultured in the authors' laboratories, none has become 'immortal', or lost either attachment dependence or growth inhibition at confluence. Also, cellular inoculation at other sites is not analogous physiologically to inoculation in the skin, and therefore, results obtained at different sites may not accurately represent behavior of cultured epidermal cells applied in or beneath the skin. In addition, orthotopic grafting of cultured skin substitutes prepared from numerous other strains of keratinocytes has similarly not resulted in any tumor formation in athymic mice [5]. Furthermore, a limited patient population [6,9, 15] that have had wounds re-epithelialized with cultured skin containing keratinocytes grown in serum-free media, has exhibited no epithelial tumors or lesions during the first year after grafting. These results agree with findings that autologous keratinocyte cell sheets do not develop neoplastic changes up to 5 years after grafting [8].

These findings of this study support strongly the postulate that transformation of HK in culture is no greater than the spontaneous rate of transformation for HK in normal epidermis. These findings also suggest that the rapid growth rate of HK cells in culture results only from permissive growth conditions and absence of inhibitory regulatory factors that stimulate the increased mitotic rate associated with the normal processes of wound healing. Based on these findings, we conclude that rapid expansion of HK cultures for the purpose of transplantation in the treatment of burns presents no detectable risk of malignant transformation to patients.

Acknowledgment

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