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EXPLORATORY STUDY ON THE STABILITY CHARACTERISTICS OF COMMERCIAL HUMAN KERATINOCYTES

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The purpose of this study was to determine the suitability of commercially available human keratinocytes for the initiation of in vitro human epidermal organotypic models for vesicant research. Commercial normal human abdominal epithelial keratinocytes were culture in vitro in serum free growth medium in sterile plastic culture flasks. The morphology, ultrastructure, growth and viability of the developing serially cultured keratinocyte cell lines were observed intermittently, and documented by phase contrast and transmission electron microscopy. First passage keratinocytes appeared normal. However, many containe paranuclear vacuole and cytoplasmic inclusions. Viability, as determined by the dye exclusion method, dropped from 94% in the second passage to 62% in the fifth passage. A total of 17.73 population doublings were obtained between the first and fifth passage (exclusive of the primary passage). Data obtained showed that senescence and differentiation occurre in the third passage. No evidence was observed for the presence of stem cells in the cell lines. This study established that commercial abdominal keratinocyte cultures were useful through the second passage for short-term experimental assays, but not for long-term

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PREFACE

This protocol was performed to determine the suitability of commercially available human keratinocytes for the initiation of in vitro reconstructed human epidermis (RHE) and human living skin equivalent (LSE) organotypic model systems in protocols for vesicant research.

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****************TABLE OF CONTENTS****************

PAGE
LIST OF FIGURES..vii
LIST OF TABLES...vii
INTRODUCTION...1
MATERIALS AND METHODS....................................1
Keratinocyte culture.....................................1
Subculture...2
Electron microscopy......................................3
Definitions..3
Total and viable cell counts.............................4
Estimation vs. quantitation of culture confluency........4
Relative cell size estimations...........................4
RESULTS..4
Morphological observations: Phase contrast microscopy....4
Ultrastructural observations: Transmission electron microscopy...5
Cell viability and growth................................6
Culture confluency estimations...........................6
Relative size of abdominal keratinocytes in culture......6
DISCUSSION...7
CONCLUSIONS...10
REFERENCES..23
LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS..............29
DISTRIBUTION LIST.......................................31

LIST OF FIGURES AND TABLES

Figure Page

1. Viability of Commercial Abdominal Keratinocytes NHEK 46 in Serial Culture 11
2. Viability of Commercial Keratinocyte Line NHEK 46 as Reculture Cell Lines 12
3. Relationship Between Commercial Keratinocyte Cell Line Population Doublings and Passages 13
4. Relationship Between Total Cell Count and Percent Confluency in Serial Passaged Cell Lines 14
5. Relationship Between Total Cell Count and Percent Confluency in Reculture Passaged Cell Lines 15
6. Micrographs of Serial Passaged Human Abdominal Keratinocyte Growth Cultures 16
7. Electron Micrographs of Serial Passaged Human Abdominal Keratinocyte Cells 17
8. Light and Electron Micrographs of Differentiated Human Abdominal Cells and Colonies 18

Table Page

1. Commercial Abdominal NHEK Morphology 19
2. Culture Growth Confluency Estimates 20
3. Relative Sizes of Abdominal Keratinocytes in Culture 21

INTRODUCTION

This study of in vitro grown secondary culture commercial human keratinocytes was conducted to assess their utility for developing organotypic skin models for vesicant research. It has been shown that such in vitro models (e.g., reconstructed human epidermis [RHE] and living skin equivalents [LSE]) require normal keratinocytes (basal cells; stratum germinativum) that can proliferate long-term without loss of either viability or morphological integrity (1, 5, 10, 22, 31). Normal human neonatal foreskin keratinocytes have an in vitro finite lifespan of about 40-50 population doublings in serum free media (6, 16, 46). It has been shown that the inverse relationship between cell growth response in low calcium growth media and cell differentiation expression in high calcium media does not alter keratinocyte finite lifespan (14, 17, 46). Additionally, it is essential that no/few abnormal basal cells occur during the cultivation period because the in vitro skin organotypic models are destined to be exposed to alkylating agents which are both toxic and carcinogenic.

Commercial human keratinocytes have been used increasingly in cytotoxicity assays (2, 3), ocular irritancy prediction assays (39), gap junction function and intercellular communication experiments (24, 36), cellular adhesion (20), cell differentiation studies (19), and growth optimization in defined media for cytokine studies (30). Several commercial keratinocyte kits were received and used in this laboratory for vesicant cytotoxicity assays. It was determined that every keratinocyte culture received had many heavily vacuolated cells regardless of site of epidermal origin, and these cells did not appear normal by transmission electron microscope (TEM) analysis. It was confirmed previously that many of the vacuoles were lipid droplets (SBO, Sudan Black B stain; and Paul T. Wegener, Clonetics Corporation; personal communications), but considered by one of us (JPP) not to usually occur in a cell line isolated from normal human neonatal foreskin. Data from this study will be used to determine the utility of normal commercial keratinocytes to initiate and sustain in vitro human skin organotypic model systems.

MATERIALS AND METHODS

Keratinocyte culture. Duplicate EpiPack Kit keratinocytes were handled and treated upon receipt as specified in the accompanying instruction insert (Clonetics Corporation, San Diego, Ca. Instructions for EpiPack containing NHEK 46, Lot # 00762 Abdominal Keratinocytes. 1989). Culture flasks were unpacked and decontaminated immediately with 70% alcohol. The flasks were equilibrated at 37°C for several hours before replacing the shipment solution with 5.0 ml of warm keratinocyte growth medium (KGM). The media and techniques recommended by Clonetics were developed by Ham and Associates (6, 7, 8, 26, 27,

41). KGM is a serum free modification of MCDB 153 medium to which whole bovine pituitary extract (wBPE; 70 ug/ml) was aseptically added after receipt. The medium was pH 7.4, 340 +/- 4 mOsm/kg, and contained 0.15 mM Ca^{2+} (Sandra L. Weise, Clonetics Corp., personal communication). Both cultures were examined by phase contrast inverted microscopy (PCIM, Zeiss) for signs of distress or deterioration. No specific tests were performed for the presence of mycoplasmal contamination of the cell lines. One flask was retained for serial culturing. The second flask of cells was prepared for TEM.

KGM was changed three times a week with 5.0 ml/25 cm^2 of warm medium in a laminar flow biohood (BiochemGard model, The Baker Company, Inc., Sanford, ME). Culture flasks, with screw caps loosened one-quarter turn, were incubated in a humidified atmosphere at 37°C (8) and 5% CO_2 (Model 3332, Forma Scientific, Marietta, OH). Keratinocyte cultures were observed for morphological and ultrastructural changes intermittently under standard conditions of serum free growth medium and incubation. The appearance of unusual numbers of paranuclear vacuoles (PNV), inclusions, pleomorphism, or ultrastructural changes in the cells were recorded and photographically documented. Particular notice was taken of cultures which contained 10% or more of nonviable cells, numerous vacuolar inclusions, and/or displayed unusual ultrastructural and pleomorphic characteristics. Subcultures were performed when cell surface growth became 50-80% confluent.

Subculture. The commercial abdominal keratinocyte culture, NHEK 46 Lot # 00762 (Clonetics), was subcultured (serially cultured) up to an additional six passages following receipt. Data were recorded for as many cell line cultures as possible in every passage. Subconfluent serial culture flasks were "recultured" in the first, second and third passages immediately following routine subculture procedures. In these instances, KGM was added to cells remaining in the subcultured flask, and the flask reincubated, to determine if any significant differences in growth parameters would occur in the ensuing reculture cell lines. This procedure was based on data showing that a primary human mammary epithelial culture could be partially trypsinized six or more times, and still yield secondary cultures having more active growth than tertiary serial cultures (40).

Before initiating subculturing procedures, PCIM micrographs were taken of each culture flask with a Contax/Yashica model 137 MA Quartz camera (Contax/Yashica, Paramus, N.J.) on Panatomic-X 32 film (Kodak, Rochester, N.Y.). One culture flask (25 cm^2 flask) of each representative pair/set of a passaged cell line was processed and analyzed by TEM (see below). Conditioned (spent) culture medium was aspirated from the remaining flask(s), and the cell monolayer washed with 2 ml HEPES buffered saline solution (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HEPES BSS) (Clonetics). The cells were covered with 2 ml of 0.025% trypsin/EDTA solution (Clonetics), and about half the volume removed 30-60 seconds later. The flask was incubated (37°C) with the cap tightly sealed. The cell monolayer was

examined at 3- to 4-minute intervals by PCIM to determine when 50% of the cells had detached. A gentle rap against a hard object released almost all cells from the culture surface. Two milliliters of Trypsin Neutralizing Solution (TNS) (Clonetics) were added immediately, and the cell suspension transferred to a sterile glass centrifuge tube. The flask was rinsed with 2 ml more of TNS to recover additional cells. The cell suspension was centrifuged at 220 xg for 10 minutes. The supernatant was aspirated and the pellet gently resuspended in 1-5 ml of warm KGM. Culture flasks, centrifuge tubes, and other cell culture paraphenalia were decontaminated with bleach.

Total cell and viable cell counts were performed for cell suspensions using nigrosin dye (EM Science, Cherry Hill, N.J.) and a coverslipped hemocytometer. Cell suspensions were used to initiate two or more new culture flasks/passage. New culture flasks were inoculated at densities ranging from 240-10,800 viable cells/cm^2, and incubated as described. The culture medium was changed at 24 hours, and the cultures examined by PCIM for contamination and to determine that at least 90% of the cells were attached to the flask surface. The cultures were observed microscopically every 24 hours for evidence of proliferation (mitotic figures) and/or changes, and the medium changed three times a week. When the growth in sibling flasks became 50-80% confluent, one was selected for serial subculturing, and the other for TEM processing and analysis. Cell line culture, subculture, and documentation procedures were continued until growth was no longer evident.

Electron microscopy. All but a thin layer of conditioned culture medium was removed from a near confluent flask. Sterile plastic scrapers were used to gently remove monolayer cell growth from the surface of the flask. Scrape-harvested cells were centrifuged at 250 xg and the supernatant medium decanted. The cell pellet was treated by routine procedures used to prepare isolated cell samples for TEM observations (28). Cells were fixed and embedded in epoxy resin (Polysciences, Inc., Warrington, PA). Thin sections (LKB Ultratome model 2128, Pharmacia LKB Biotechnology, Piscataway, N.J.) were stained nonspecifically with lead citrate and uranyl acetate solutions (LKB Ultrastain model 2168, Pharmacia LKB), and analyzed by TEM (Model JEM-1200EX, JEOL, Peabody, MA).

Definitions. Serial cultures in this report refer to the routine manner in which cell line cultures were consecutively passaged (subcultured) using a known viable cell count inoculum. A reculture cell line was initiated when a routinely subcultured flask, still containing an unknown number of viable cells, was incubated with fresh media, and subsequently routinely cultured and subcultured. All cultures had finite lifespans in in vitro culture to a population doubling level of 17.2 in five passages over 40-50 days, excluding primary culture. Aside from the above definition for reculture cell lines, this report adheres to accepted cell culture terminology (38).

Total and viable cell counts. Cells were stained with nigrosin dye (21), and total cell and viability counts were performed by procedures described previously (9). Quantitative calculations were obtained for total cell count, viable cell count, percent viability, population doublings (PD) (38), and population doublings/day (PD/D) from the cell count data.

Estimation vs. quantitation of culture confluency. Subjective estimates of culture flask cell growth confluency were used throughout this study to determine relative growth, and to initiate subculture procedures. To gauge the accuracy of recorded subjective estimates, two types of quantitative analyses were performed on enlarged positive photographic prints of representative culture flask growth confluency fields. The first method was an image analysis system program (Quantimet 970, Cambridge Instruments, Cambridge, MA). In the second method, photographs were weighed, cell areas excised, weighed, and the area occupied by cells expressed as percent of total weight.

Relative cell size estimations. Relative cell line sizes were estimated from total count and percent confluency data as numbers of cells per 1.0% confluency. Relative cell size (RCS) was determined for each serial and reculture cell line, and between serial and reculture cultures of the same passage.

RESULTS

Morphological observations. Phase contrast microscopy. First passage cells were slightly rounded after several hours of incubation following receipt of the culture. Over 90% of the cells attached after 24 hours incubation with some round cells (presumed dead) still present. The abdominal cell population contained a heterogeneous mixture of columnar, spindle, and cuboidal shapes associated with normal epithelial keratinocytes. Ovate and columnar forms containing large nuclei were predominant early in the first and second passages. Pleomorphic forms were also prominent at this time (Table 1; Fig. 6B). Cells initially attached to the plastic culture flask surface, and grew alone or in loosely associated clusters. Constant surveillance from one observation to another confirmed that individual cells migrated to open areas. Many keratinocytes, alone, or in small clusters, had ruffled membranes (not shown), consistent with migratory activity at this stage of culture growth (25). In 2-3 days patches of nonconfluent cluster colonies contained more polygonal forms as the culture density increased, with many cells having paranuclear vacuoles (Fig. 6A). Granular, particulate inclusions were seen in some cells in these passages. Second and third passage serially cultured cells had similar growth and tight polygonal cluster-colony patterns (Fig. 6B).

Fourth passage cell lines contained increased numbers of paranuclear vacuoles and showed a slight morphological change from, and loosening of, the tight polygonal growth pattern (Fig. 6C). Fifth passage cells increasingly lost their polygonal colony appearance, and more unusual epithelial cell shapes appeared

(Figs. 6D, 8A). Terminal differentiation occurred in the sixth passage where nuclear disintegration, cellular necrosis and cellular disintegration were seen (Fig. 8B).

Giant cells were conspicuous in early passages (Figs. 6B, 6C), and persisted to the end of the growth phase (fifth passage) when they were reduced by 99% (Table 1). Elongated needle shaped cells were seen throughout the lifespans of all cell lines derived from the initial culture (Table 1; Figs. 6B, 8A). Double nucleated cells occurred randomly in all passages (Figs. 6C, 7C), and were not considered an anomaly because of their occurrence in normal tissue. Paranuclear vacuoles were present during the lifespans of the cell lines (Table 1). Nuclei with 1-3 paranuclear vacuoles were prominent in early and late passages (Table 1; Figs. 6A, 8A). Nuclei with 4+ paranuclear vacuoles were prevalent in the second passage, and after a sharp decline in passages 3 and 4, gradually increased in subsequent passages (Table 1; Figs. 6C, 6D, 8A). Cell size decreased slightly as the serial cultured cell lines entered senescence (third and fourth passages), but became large flat cells in the sixth passage, the terminal differentiation phase (Table 3). Conversely, cell size of the reculture cell lines increased markedly between passages 4 and 5 (Table 3). Stratification very rarely occurred, and when present was limited to pinpoints on areas of dense growth in nonconfluent culture flasks (not shown).

Ultrastructural observations. Transmission electron microscopy. TEM micrographs taken at each passage showed abdominal keratinocyte populations to be predominantly normal cells during the early passages. First and second passage normal abdominal keratinocytes had a rounded dense nucleus, distinct nucleoli, intact rounded nuclear membrane components, large prominent microvilli, endocytotic vacuoles, an abundance of mitochondria, rough endoplasmic reticula, and a ring of perinuclear tonofilaments (Figs. 7A, 7B). Furthermore, there was no evidence of desmosomal buildup or attachment sites (gap junctions) at the cell membrane as was reported previously for keratinocytes in low calcium serum free medium (11, 12, 18, 25, 30, 34, 39, 41).

Increased numbers of endocytotic vacuoles, and the appearance of many lipid droplets and lysosomes occurred in the third passage (Fig. 7C). Further evidencef senescence was seen in the fourth passage, which showed the onset of nuclear membrane indentation, rarifying cytoplasm, smaller microvilli, and increasing numbers of lysosomes and lipid droplets (Fig. 7D). Senescence was firmly established in the fifth passage by lipid droplet paranuclear vacuoles severely indenting an already invaginated nuclear membrane in the presence of increased cytoplasmic lysosomes and lipid droplets, and numerous but less prominent microvilli. Paranuclear vacuoles indented the nuclear membrane in such a mannner that they had the appearance of being intranuclear at low magnifications, as shown in Figs. 6B and 6C. Terminal differentiation occurred in the sixth passage where the nucleus was rarified and devoid of nucleoli, its nuclear membrane

convoluted, and membrane components detached in several places (Fig. 8D). Additionally, the area around the nucleus was also rarified, with myelin figures and lysosomes more prominent. Many mitochondria were devoid of substance and appeared almost empty.

<u>Cell viability and growth</u>. Serial culture cell lines were highly viable in the first and second passages (>90%), but dropped below 90% from the third (76%) to the seventh passage (0%). The sixth passage results were an anomoly of the cell counting procedure (Fig. 1). Conversely, reculture cell line viability never exceeded an average of 87% (Fig. 2). Maximum average population doublings/day (PD/D) occurred in the third passage in serial cultured cell lines (0.54), and decreased to zero by the sixth passage (Fig. 1). Reculture cell lines averaged 0.15 PD/D in the third passage, and dropped to 0.03 PD/D in the fifth passage (Fig. 2). Total PD for serial cultured cell lines 1E and 1G were 17.73 and 16.65, respectively, beginning with the first passage (Fig. 3). Total PD for cell lines 1E and 1G occurred by the fifth and fourth passages, respectfully, in 50 days or less (Fig. 3).

<u>Culture confluency estimates</u>. The decision to subculture or terminate a particular culture flask was dependent upon a subjective estimation of the culture growth confluency covering the surface area. The reliability of the subjective estimates was paramount for analytical considerations. A comparison between the subjective and two quantitative methods estimates was performed on representative cell lines from several passages. Total cell counts/culture flask for serial and reculture cultures remained constant through the fifth and fourth passages, respectively, and declined thereafter (Figs. 4, 5). Serial and reculture culture confluency declined in the third and fourth passages, respectively (Figs. 4, 5). There were no significant confluency differences for the seven samples at 95% C.I. between the quantitative and subjective methods estimates (Table 2).

<u>Relative sizes of abdominal keratinocytes in culture</u>. Abdominal keratinocytes in serial cultured cell lines maintained an average relative size of 0.76 (passages 2-5) with respect to the initial culture line (Table 3, first passage). However, the general tendency was toward successively slightly smaller sizes in these cell lines, until the sixth and seventh passages, when the sizes increased perceptively (data not shown).

Keratinocytes derived from reculture cell lines showed a markedly increased cell size with each succeeding passage (Table 3, passage 3). The average relative reculture keratinocyte size doubled over the previous cell size in passages four and five. These data agree with results reported by some investigators who considered enlarged cells as the initial differentiation or senescence signal (23, 47), or as the first stage of terminal differentiation (12).

A comparison of relative sizes between serial culture and reculture cell lines showed that the latter were larger overall, especially in passages four and five (Table 3).

DISCUSSION

As a result of this study we found commercial abdominal keratinocyte cultures to be normal, proliferating cell lines through their second passage. Reduced viability, and cellular ultrastructural changes signalling the onset of differentiation, senescence, and ultimately terminal differentiation started in the third passage and ended in the sixth passage.

The majority of references describing the establishment of primary and secondary keratinocyte cell lines were initiated with human circumcised neonatal foreskin tissue. They include the research upon which the commercial keratinocyte production procedures were based (7, 8). Published data for keratinocyte cultures from human neonatal foreskin tissue showed population doublings/day of 0.90 in the second passage, and about 40 PDs/lifespan (6, 26). The highest proliferation rate obtained with abdominal keratinocytes was 0.54 PD/D in the second passage (Fig. 1), with a total of 16-17 PDs from passages one to five (Fig. 3), not including the estimated primary passage data. One possible reason for the difference is that the commercial abdominal cells used in this study were obtained by elective surgery from adult donors of various ages. Several investigators have reported that the skin site origin of primary cultures, and ages of the donors may have a bearing on cell response during cultivation under equivalent conditions (13, 15, 35, 42). On the other hand, Briggaman et al. (9) in an earlier study reported essentially no difference due to human keratinocyte body origin. It should be noted, however, that neither Briggaman et al. (9) nor Hawley-Nelson et al. (15) included human abdominal cell cultures in their investigations.

Evidence presented in this report for relative cell sizes indicates that average serial cell line cultures were fairly constant in size through the fifth passage, whereas the average reculture cell lines became markedly larger with subculturing (Fig. 4). The latter were considerably larger relative to the former, especially in passages 4 and 5 (Fig 4). Boyce and Ham (6) reported that relative cell size increased with each succeeding passage, as was seen in our reculture cell lines, but not in serial cultures. Cell enlargement has been identified as one potential signal for differentiation, senescence, and terminal differentiation (6, 12, 26, 44, 46). Stampfer et al. (40) regarded increased cytoplasmic vacuolization as the initiation of senescence (e.g., increased endocytotic vacuoles, lipid droplets, and lysosomes in the third passage, Fig. 7C). It was reported that the proliferation potential of keratinocytes was inversely correlated with their size (4). Using this criterion with respect to our data (Figs. 3-5) and the first passage culture (Table 5), there was little or no change in the cell size or growth activity of serial cultures from passages 2-5, whereas all the reculture cell lines were increasingly larger on average, and less active.

Cell viability and morphological integrity were important considerations in this investigation, in deference to other cell culture parameters. Abdominal epithelial keratinocytes were confirmed in this study by their morphology by light and electron microscopy. A modification of the dye exclusion cell viability count method (9, 21) was used in this study as a measure of cell physiological potential for proliferation. The procedure is rapid, and had been shown to be capable of detecting dying as well as dead cells (45). The only stress placed on the exponentially growing cells prior to the dye exclusion procedure was a trypsinization procedure at 40-85% of cell culture confluency. The dilute trypsin treatment has been shown to adversely affect dead and dying cells, in that the former are digested and the latter damaged further. Physiologically healthy cells were unaffected except for a minimal permeability which resulted in some cells slowly taking up the dye (29). Abdominal cells were 100% viable in the initial subculture (first passage), but dropped below 90% in the third passage, and continued to decline until terminal differentiation by the fifth (reculture cell line) and sixth (serial cell line) passages (Figs. 2 and 1, respectively). The dye exclusion procedure for viability determination was probably more reliable in the first few passages where the cells were more resistant to trypsinization procedures.

Stampfer et al. (40) described a procedure in which a primary culture of human mammary keratinocytes grown and maintained in a medium containing 5% fetal bovine serum (FBS) was partially trypsinized and secondary cultures established periodically. A comparison of secondary culture growth curves vs. routine tertiary serial cultures, also derived from the original primary culture, showed that the secondary cultures grew 2-5 times more actively than the tertiary serial cultures. Six secondary cultures were prepared from the primary culture over about as many weeks. Since Stampfer et al. (40) were able to successfully retrypsinize their primary culture persistently, it suggested the possibility of stem cells being present in the primary culture and perhaps early subcultures. Watt (43) inferred from cultured normal skin grafts placed on burn victims that persisted for long periods, that stem cells existed at least in early culture passages. It appeared that the predestined history of normal keratinocytes could be abrogated, and the decline of cell growth and viability beginning with the third passage together with the initiation of differentiation and senescence, could be circumvented or deferred before that time. The reculture experiments were an attempt to test the hypothesis with first, second, and third passage recultures. However, experimental results failed to confirm the hypothesis in these passages because growth rate and general viability were lower in reculture cell lines, and terminal differentiation occurred one passage earlier than in the serial cultures. Apparently stem cells removed from primary culture and subcultured in vitro become inactive, thus permitting senescence to occur.

Alternatively, it has been suggested that cell-cell interaction (40) and/or gap junctional intercellular communication (43) may be present in primary cultures but sometimes lost in secondary cultures, at or before terminal differentiation. The physical association of cells at subculture (i.e., individual vs. clumps of cells) (40). and/or the content of the growth medium (24) may determine loss or retention of primary culture attributes.

Colonies grew unevenly and merged spottily up to 75% of confluency, after which cells became flat, even monolayers. Abdominal cell populations had the general morphology of normal epithelial keratinocytes in vitro (18), as well as from specific areas of human skin (e.g., neonatal foreskin: 23, 25,32, 46; mammary: 37, 40; cervical: 42, 47; tracheobronchial: 11). About 80% of the cells displayed a normal morphology during the lifespan of the cultures (Table 1). Giant and needle cells averaged 6.3% in all passages, but were prominent mostly in earlier passages.

Paranuclear vacuoles persisted throughout the lifespan of the cultures (average of 14%), but nowhere in the literature were they specifically mentioned. Close scrutiny of pictorial illustrations in published papers failed to disclose them. Similar vacuoles were seen previously in our laboratories in human breast keratinocyte cell lines from the same commercial source. Paranuclear vacuoles (as storage vacuoles and/or lipid droplets) may be a consequence of the serum free medium in which the cells were grown, and as such, considered "normal". However, Ponec et al. (32) showed that lipogenesis was lowest in well-differentiating cell lines and normal keratinocytes. Lipid droplets were observed (33) in epidermal hyperproliferative states (e.g., air-liquid interface culture and psoriatic epidermis). The normal abdominal keratinocytes in this study were grown in vitro submerged in an optimal serum free growth medium (6). An increase in lipid droplets occurred in the third passage (along with the appearance of endocytotic vacuoles and lysosomes) clearly indicating the initiation of differentiation. Paranuclear vacuoles apparently had no effect on the viability or proliferation of cultured cell lines.

Pinpoints of stratified, differentiated cells occurred rarely, and only in densely populated areas of the culture flasks. These cells stained lightly with nigrosin, and were not rounded like other trypsinized cells. They were not included in the total cell count. Perinuclear keratohyalin granules, another differentiation marker, were seen ultrastructurally in later passages of the cultures (data not shown).

The absence, or undetected occurrence of gap junctional intercellular communication sites (GJIC) in our culture cells was probably due to the presence of EGF, and/or wBPE in KGM at relatively low calcium levels. EGF at 10 ng/ml in serum free KGM, like similar mitogens, was shown to inhibit GJIC formation (24).

CONCLUSIONS

Commercial abdominal cultures attain a useful zenith through their second passage, after which growth rate, viability, and cell integrity decrease; but differentiation, senescence, and terminal differentiation start in the third or early fourth passage. There are still aspects of commercial keratinocyte cell lines that are unexplained (e.g., the presence of paranuclear vacuoles, giant and needle cells, and the possible difference between cell lines originating from different skin sites).

Based on results of this study and the literature, commercial keratinocytes can be confidently used for short term toxicological type assays in the first and second passages, but with reduced reliability in the third passage and beyond. This view is supported by the vendor who does not assure the quality of the product beyond the second passage.

Commercial keratinocytes were shown to have expended about 40% of their population doublings by the end of the first passage. These represent, together with the second passage, the prime of the cell culture lifespan. Starting with the third passage, serially subcultured cell lines declined in growth rate, viability, and morphological integrity, in agreement with most published data. Experimental data were cited (40) which showed that when primary cultures were repeatedly 50% trypsinized and the detached cells subcultured, the primary culture could potentially become an unlimited supply of actively proliferating secondary cultures of basal keratinocytes. Experiments to reculture early passage subcultured flasks in this study did not emulate the primary culture results cited. Possible reasons for the results are the presence of active stem cells in primary cultures, but not in in vitro subcultures. Conversely, primary culture keratinocytes were considered by some investigators to have limited proliferative potential, so they used third and fourth passage commercial keratinocytes (24). Data presented in this study do not confirm that view, but show that growth rate, viability, morphological and ultrastructural integrity were markedly greater prior to the third passage. The data support the position that skin organotypic model research should be initiated and performed with primary and/or first passage cell cultures.

Studies undertaken with commercial keratinocytes over an extended period of time should be conducted with the same keratinocyte cell product and preparation (human abdominal, breast, foreskin, etc.) to insure reliable, reproducible results.

Figure 1. Viability and growth of commercial abdominal keratinocyte NHEK 46 cell line in serial culture.

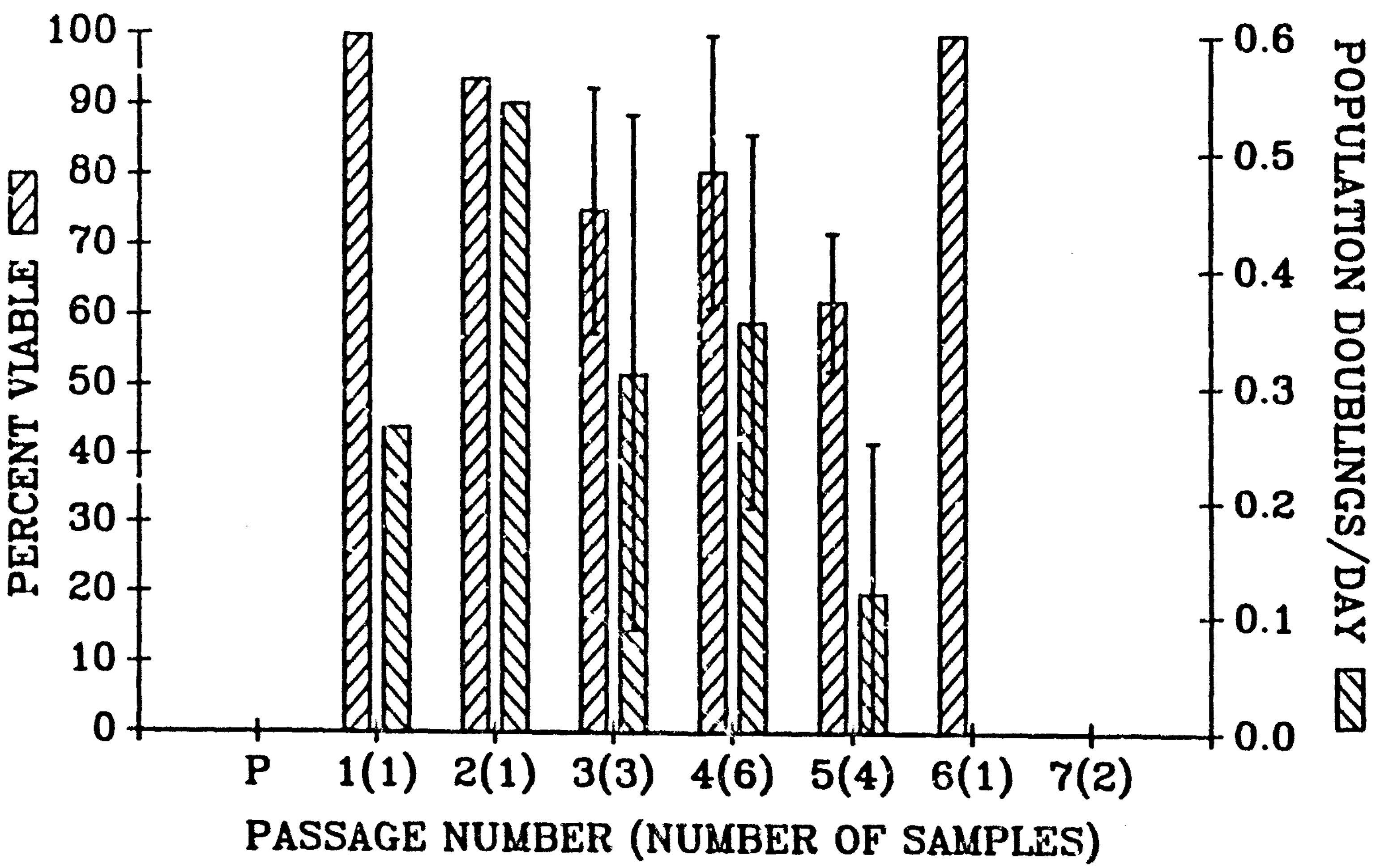


Figure 2. Viability and growth of commercial abdominal keratinocytes as reculture cell lines.

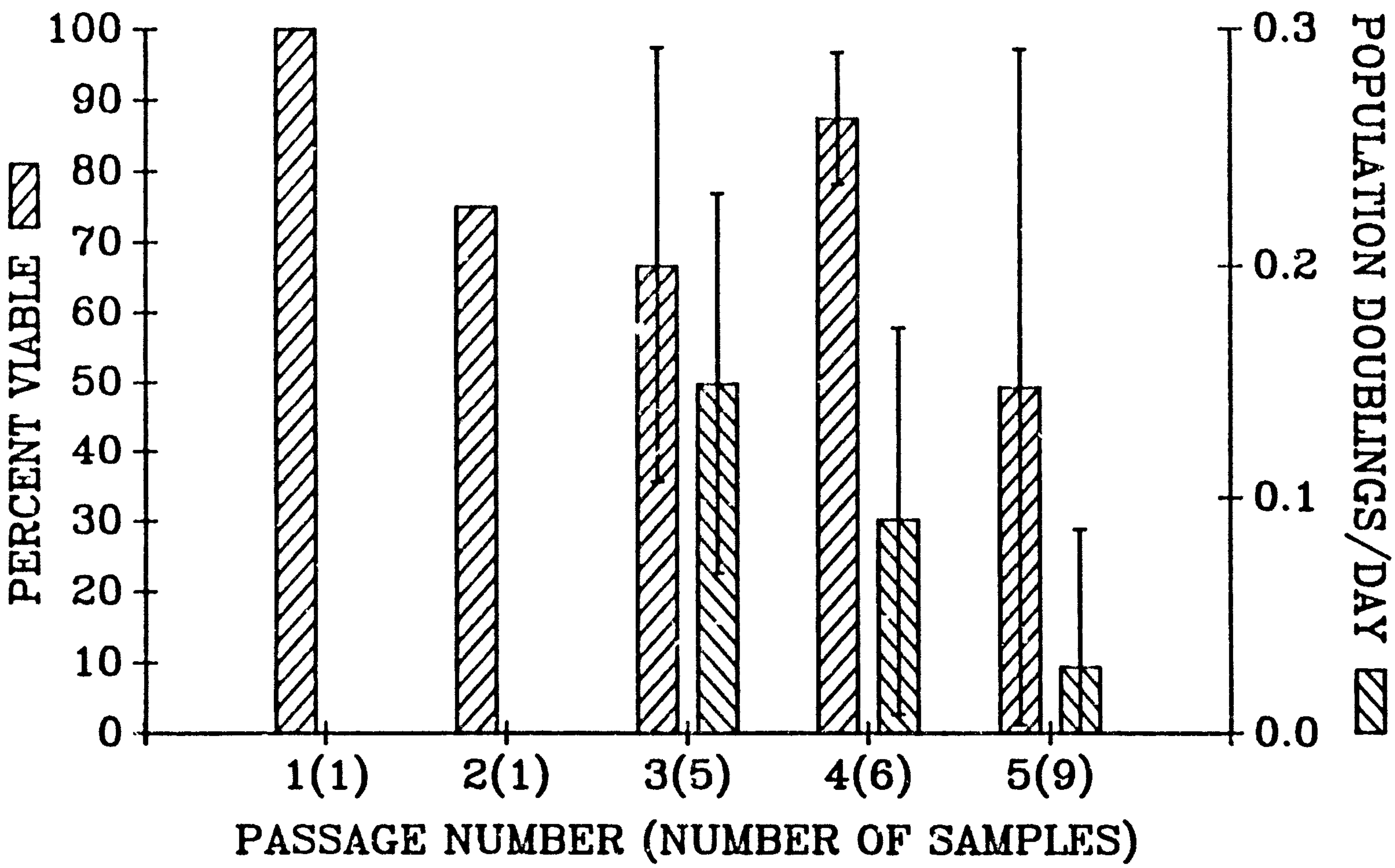


Figure 3. Comparison of two serial cell lines (series 1E and 1G) with respect to population doublings, passages, and incubation times. Cell line 1E ceased to grow after the fifth passage, and cell line 1G stopped after the fourth passage.

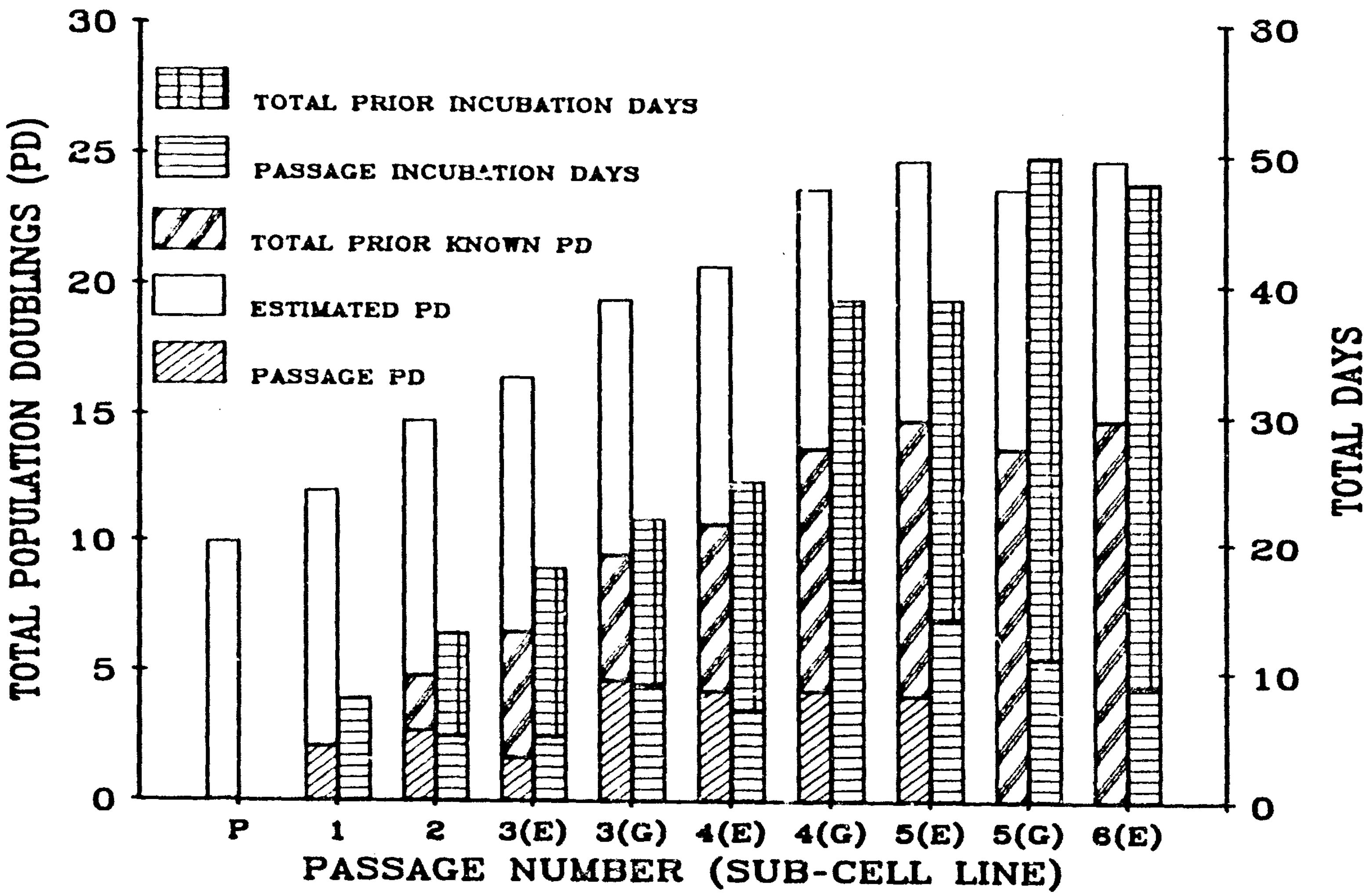


Figure 4. Relationship between total cell count and percent confluency in serial cultured abdominal keratinocyte cell lines.

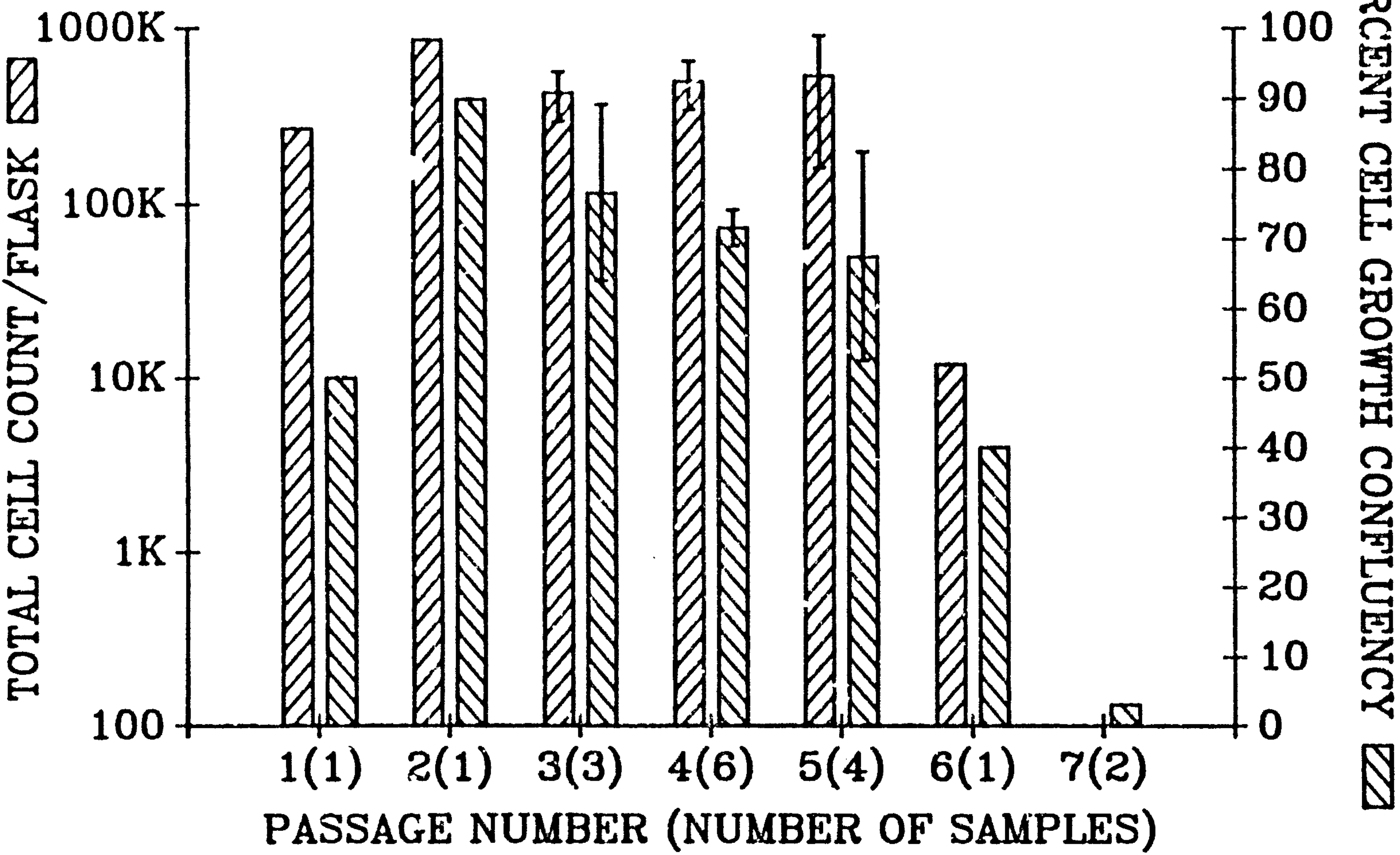
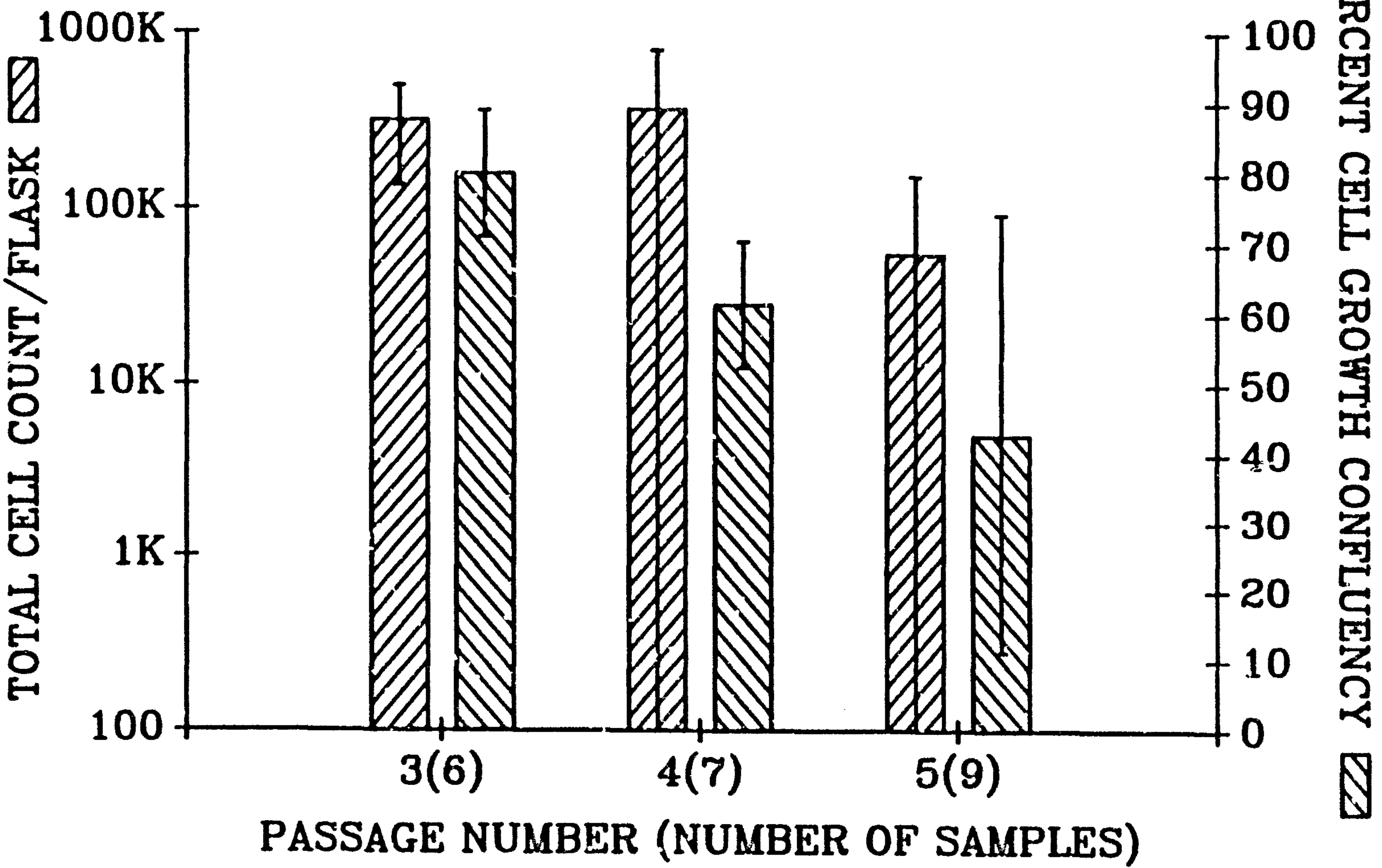


Figure 5. Relationship between total cell count and percent confluency in reculture cell lines of abdominal keratinocytes.



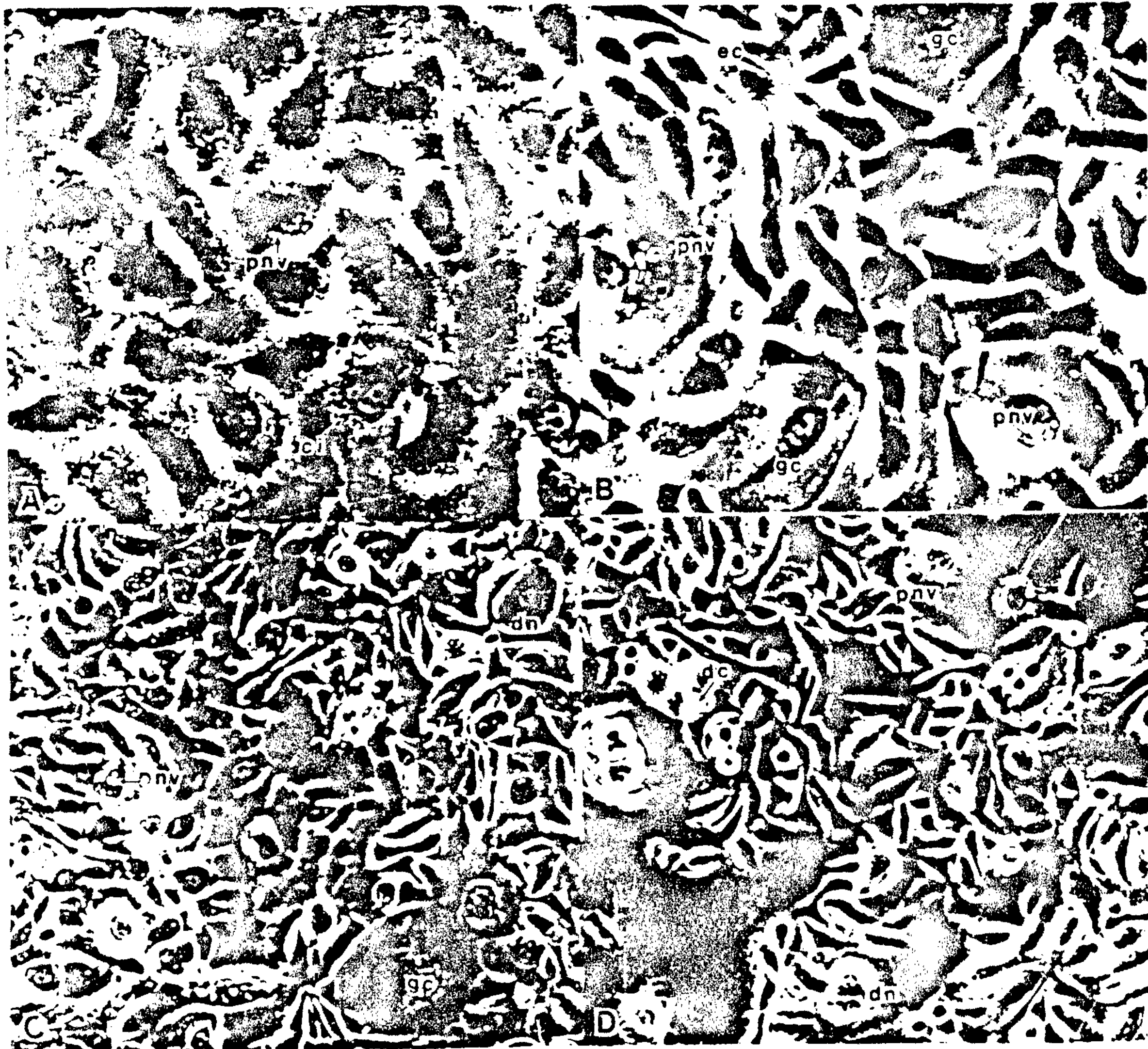


Figure 6. Micrographs of serial passaged commercial normal human abdominal keratinoc tes. A. First passage polygonal growth pattern. x1,147. B. Third passage showing some pleiomorphic forms. x717. C. Change to loosening colony growth pattern occurred in the fourth passage. x358. D. Looser colony growth and senescent appearance seen in the fifth passage. x358. ci, cellular inclusions; dc, dividing cell; dn, double nucleus; ec, elongated (needle) cell; gc, large giant cell; n, nucleus; pnv, paranuclear vacuole.

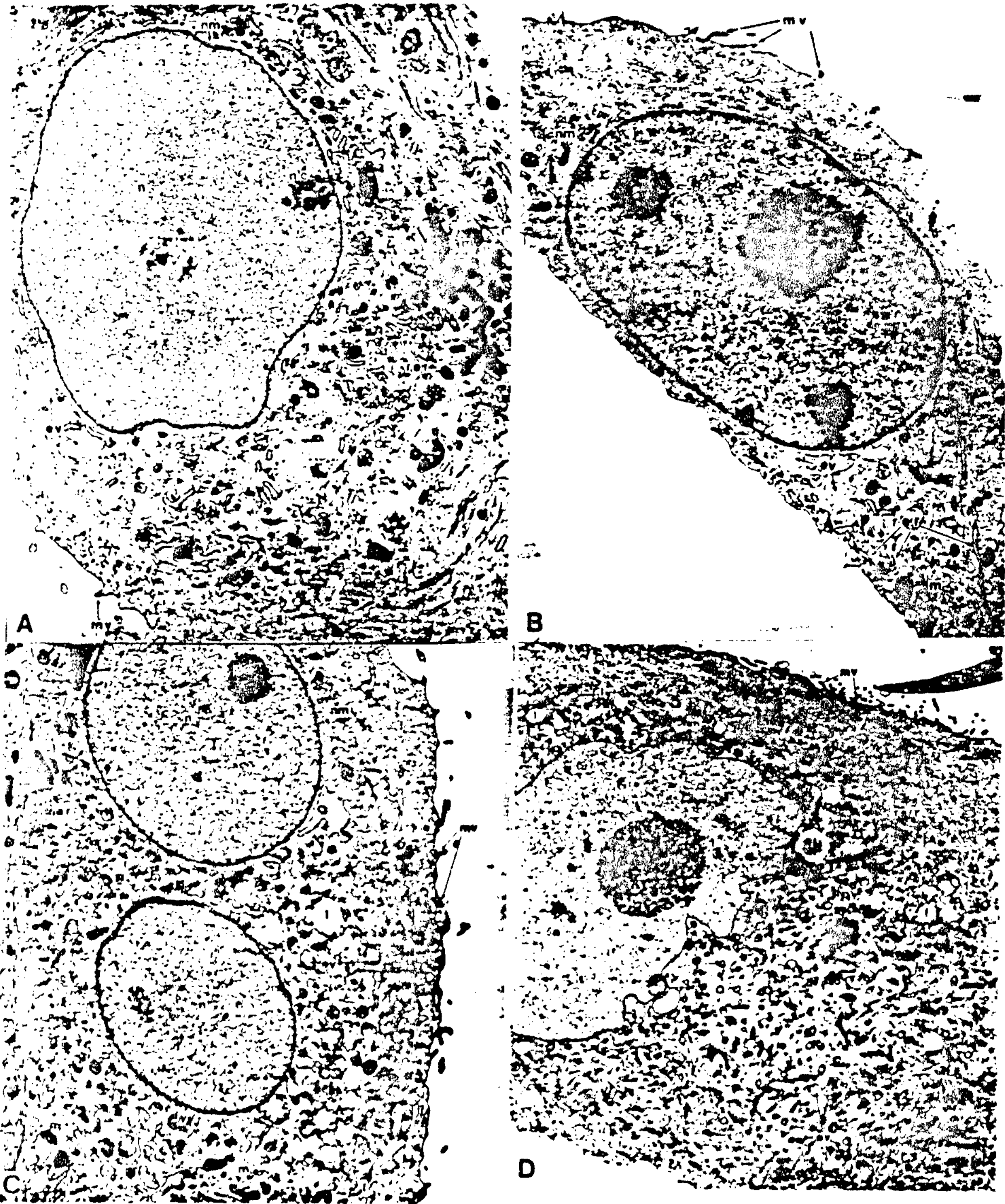


Figure 7. Transmission electron micrographs of isolated normal human abdominal keratinocytes from serial passaged cultures. A. Ultrastructure of first passage normal cell 24 hours after receipt. x9,500. B. Second passage cell from subconfluent growth culture. x8,400. C. Third passage double nucleated cell showing first signs of senescence and differentiation (increased endocytotic vacuoles and lipid droplets, and the appearance of lysosomes). x5,600. D. Fourth passage senescent cell having an indentated nuclear membrane and rarifying cytoplasm. x4,000. ev, endocytotic vacuole; l, lipid droplet; ly, lysosome; mv, microvilli; m, mitochondria; n, nucleus; nm, nuclear membrane; nc, nucleolus; rer, rough endoplasmic reticula; t, tonofilaments.

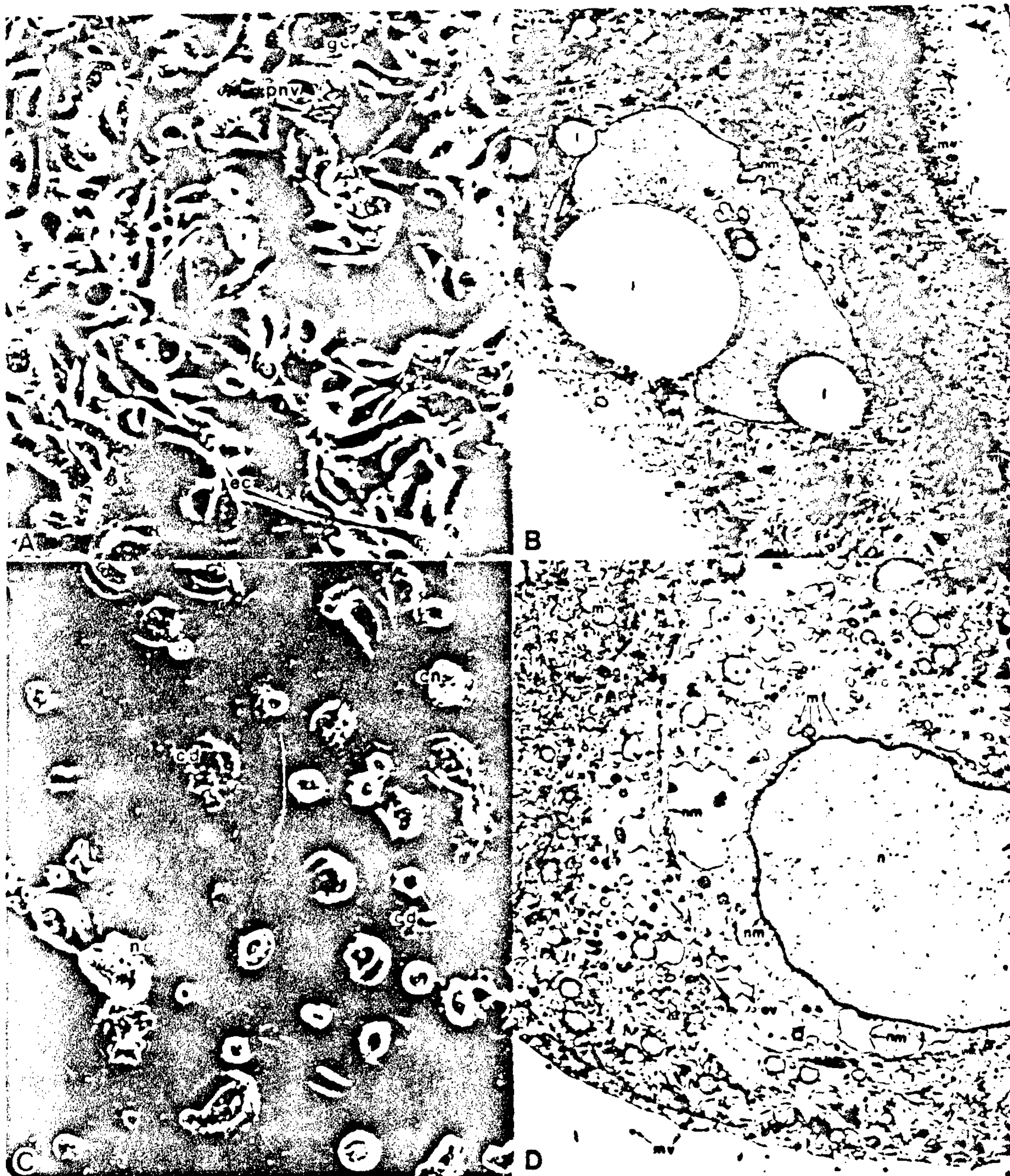


Figure 8. Light and electron micrographs of fifth and sixth serial passaged normal abdominal keratinocyte cultures. A. and C., light micrographs; B. and D., electron micrographs. A. Fifth passage growth culture showing senescent cells and colony deterioration. x358. B. Fifth passage senescent cell containing lipid droplet paranuclear vacuoles indentating a convoluted nuclear membrane. x4,500. C. Terminal phase of colony deterioration in the sixth passage. x358. D. Ultrastructure of a terminally differentiated sixth passage cell exhibiting rarified nucleus (with no nucleolus), mitochondria, and cytoplasm. Note the nuclear membrane dissociating in several places. x3,750. cd, cellular debris; cn, cellular necrosis; ec, elongated cell; ev, endocytotic vacuole; gc, giant cell; l, lipid droplet; ly, lysosomes; mv, microvilli; m, mitochondria; mf, myelin figures; nd, nuclear disintegration; nm, nuclear membrane; n, nucleus; pnv, paranuclear vacuole; rer, rough endoplasmic reticula; t, tonofilaments.

TABLE 1

COMMERCIAL ABDOMINAL NHEK MORPHOLOGY

CELL LINE CODE	PASSAGE NUMBER	TOTAL CELLS COUNTED	PERCENT					
			CELL VIABILITY	NORMAL CELLS	GIANT CELLS (GC)	NEEDLE CELLS (EC)	CELLS WITH 1-3 PNV INCLUSIONS	CELLS WITH 4+ FNV INCLUSIONS
1	1	41	100.00	85.9	9.8	2.4	17.1	4.9
1A	2	110	93.75	76.4	9.1	4.5	10.0	0.0
1E	3	353	55.56	89.8	4.0	2.5	3.1	0.1
1E-3	4	232	86.67	78.0	1.7	1.3	11.2	7.8
1G-4	4	455	90.24	84.8	3.5	1.5	7.7	2.4
1E-5B	5	297	73.58	85.5	1.0	2.4	7.7	3.4
1I-2	5	136	50.00	77.2	0.1	0.0	18.4	3.7
N			7	7	7	7	7	7
MEAN			78.5	79.7	4.2	2.1	10.7	3.2
ST.DEV.			19.4	7.9	3.9	1.4	5.4	2.7
S.E.M.			7.3	3.0	1.5	0.5	2.1	1.0

Commercial human abdominal keratinocyte morphology in culture. Cell cultures were phase photomicrographed in situ in each passage, and cells of typical cultures counted and classified.
pnv = paranuclear vacuole.

TABLE 2

CULTURE GROWTH CONFLUENCY ESTIMATES

CULTURE LINE CODE	PASSAGE NUMBER	% ESTIMATED CONFLUENCY	% CONFLUENCY PHOTOGRAPH	% DIFFERENCE PHOTOGRAPH	% CONFLUENCY I. A. SYSTEM	% DIFFERENCE I. A. SYSTEM
1	1	50	41.75	8.25	48.17	1.83
1	1	50	44.63	5.37	52.91	-2.91
1A	2	60	67.10	-7.10	61.53	-1.53
1K	3	75	79.44	-4.44	81.26	-6.26
1K-3	4	50	43.75	6.25	49.66	0.34
1G-4	4	95	97.33	-2.33	97.65	-2.65
1K-6B	5	95	96.09	-1.09	97.61	-2.61
N		7	7	7	7	7
MEAN		67.9	67.2	0.70	69.8	-1.97
ST.DEV.		20.6	24.5	5.91	22.0	2.59
S.E.M.		7.8	9.3	2.23	8.3	0.98
95% C.I.		48.8-86.9	44.5-89.8	-4.80-6.20	49.5-90.2	-4.36-0.42

Estimates of culture confluency. Enlarged phase photomicrographs were analyzed by two quantitative methods. N, number of culture flasks; Mean, mean % confluency of sample flasks; St.Dev., standard deviation; S.E.M., standard error of the mean; 95% C.I., 95% confidence interval; I.A. System, image analysis system.

TABLE 3

RELATIVE SIZES OF ABDOMINAL KERATINOCYTES IN CULTURE

PSG #	SERIAL CULTURES			RECULTURE CULTURES			SER M / REC M
	N	M(S.E.M.)	$PSG(M)_i$ / $PSG(M)_1$	N	M(S.E.M.)	$PSG(M)_i$ / $PSG(M)_2$	
1	1	5.40(-)	1.00	-	NA	NA	NA
2	1	9.60(-)	0.56	1	4.80(-)	1.00	2.00
3	3	5.28(0.64)	1.02	6	4.22(1.05)	1.14	1.25
4	6	6.98(0.75)	0.77	5	2.07(0.49)	2.32	3.37
5	4	7.68(2.04)	0.70	7	0.99(0.49)	4.85	7.76

Relative sizes of abdominal keratinocytes of serial vs. reculture cell lines. The data were compiled from individual culture confluency and total counts at subculture. PSG #, passage number; N, number of culture samples; M, mean number of cells (x1000) per 1.0% growth confluency; S.E.M., standard error of the mean; $PSG(M)_i$ or $_2$/$PSG(M)_1$, multiples of each passage mean size with respect to passage 1 or 2 mean size; SER M/REC M, multiples of reculture cell line mean size relative to serial culture mean size of the same passage; NA, not applicable.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

wBPE Bovine pituitary extract
EDTA Ethylenediaminetetraacetic acid
EGF Epidermal growth factor
FBS Fetal bovine serum
KBM Keratinocyte basic medium
KGM Keratinocyte growth medium
LSE Living skin equivalent
PCIM Phase contrast inverted light microscope
PD/D Population doubling/day
RCS Relative cell size
RHE Reconstructed human epidermis
TEM Transmission electron micoscope

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