SELECTIVE CULTIVATION OF EPIDERMAL KERATINOCYTES FROM NEWBORN AND ADULT SKIN IN A SERUM-FREE MEDIUM AND WITHOUT THE USE OF A FEEDER LAYER

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Abstract :

Selective cu*ltivation of epidermal human keratinocytes (HK) is important not only in investigating disorders that involve these cells, but also in therapy. In this work we report on a simple modified technique for cultivation of epidermal Hk from newborn and adult skin in which the need for conditioned medium, serum and specialized culture surfaces, e.g., 3T3 cells, has entirely been eliminated. The medium is a modified MCDB 153 to which crude bovine pituitary extract (BPE) 70 ug/ml, epidermal growth factor (EGF) 10 ng/ml, hydrocortisone 1.4 X 10-6 M, and insulin 10 ug/ml were added. The Ca++ level was 0.15 mM, however near confluence the primary culture was passaged and one secondary culture was maintained on this low Ca++ level, while the other was exposed near confluence to high Ca++ level (1.8 mM). Proliferation rate was calculated in both cultures and each mitogen was omitted from the low Ca++ medium and proliferation rate was also determined at day 3,6 and 9 of incubation. Cytospin preparation from low and high Ca++ cultures were labelled with MoAb against cytokeratin 5,14,1 and 10 (APAAP technique).*

Colonies were detected after 2 days of inoculation of the primary and secondary cultures and as the cells divided, they expanded and formed a continuous sheet. Cells left without passage underwent morphological changes characteristic of differentiation and were completely separated after 3-4 weeks as a continuous sheet. Cells of secondary cultures exposed to high Ca++ level underwent the same differentiation process and expressed cytokeratins of suprabasal keratinocytes, namely CK1 and 10.

On the other hand, cells maintained on low Ca++ grew as monolayer, were polygonal in shape and expressed cytokeratins similar to basal cells, namely CK 5 and 14. No difference in morphology or cytokeratin expression was observed between newborn and adult keratinocytes except that the former showed a significant stronger proliferation rate (mean cell cycle 22 + 1.5 h) when compared with the latter (mean cell cycle 25 + 0.9 h) ($p<0.05$). Proliferation rate markedly decreased in the absence of BPE (>50%) ($p<0.001$) whereas absence of EGF, insulin or hydrocortisone showed mild but significant decrease (<20%) ($p<0.05$). Few contaminating dendritic cells were usually seen in primary cultures, however, with subsequent passages, they disappeared and pure keratinocytes were obtained.

From the above data, we conclude that HK can be easily cultured in a serum-free medium and without the use of a feeder layer either as undifferentiated (basal) or differentiated (suprabasal) keratinocytes.

***Key words:** Keratinocyte culture - serum free medium and without 3T3 cells-Effect of growth factors.*

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Introduction :

Keratinocytes are the major epidermal cells since they constitute up to 90% of the epidermal cell population [(1)]. There is a growing interest for selective cultivation of epidermal human keratinocytes (HK) for better understanding of several dermatoses and for management of several skin lesions, such as burns and chronic non-healing leg ulcers. Early attempts to culture epidermal HK were by organ culture in which tissue viability was simply maintained in vitro or by explant culture when cell growth was from tiny pieces of skin. In both methods, pure keratinocytes were impossible to obtain [(2)]. It was only in 1975 when Rheinwald and Green utilizing irradiated mouse transformed fibroblasts (3T3) in a serum supplemented medium that HK as a single sheet could be obtained. Nevertheless, the presence of transformed non human cells and serum was a major drawback of this culture system [(3)]. In this report, we describe a simple modified technique for cultivation of epidermal HK from newborn and adult skin utilizing a serum free medium and without the use of supporting cells. We believe that this culture system can be used for several investigative and therapeutic purposes.

Materials and methods :

Normal epidermal HK were cultivated by modification of the method described by Boyce and

Ham[4]. Cells were obtained from foreskin of newborn and from skin of aJults undergoing elective abdominoplasty (2 different donors each). The skin was thoroughly trimmed from dermis and subcutis fat with a sharp scissor and cut into small pieces 5 mm each. The skin was incubated in trypsin 0.25% (Sigma, USA) overnight. The epidermis was separated and epidermal sheets were vigorously pipetted to obtain individual cell suspension.

They were grown in tissue culture flasks (Falcon 75 cm, Becton and Dickenson, Heidelberg, Germany) in 5% Co2 at 37 C. The cells were inoculated at a density of 5X104 cell/cm. The medium used was the modified MCDB 153 to which is added crude bovine pituitary extract (BPE) 70 ug/ml, epidermal growth factor (EGF) 10 ng/ml hydrocortisone 1.4x10.6M (Clonetics, San Diego, USA) and insulin 10 ng/ml (Sigma, USA). The Ca++ level was 0.15 mM. The medium was changed every 2 days. Near confluence, the cells were passaged and inoculated in sterile Petri dishes at a density of 5x103/cm and secondary cultures were maintained on low Ca++, however, near confluence one culture was switched to medium containing high Ca++ (1.8 mM) whereas the other was kept on the low Ca++ level. Cells were monitored by phase contrast microscopy with photographs taken to document microscopic morphological alterations.

Proliferation assays :

Second passage HK were used in this experiment. Proliferation rate of cells maintained on low and high Ca++ (in triplicate) was carried out in Petri dishes. Growth rate is expressed as cell cycle (population doubling) per 24 hrs from the formula. Population doubling (PD) = 10g2 (total cells harvested-colony incompetent cells/total colonies formed)

Counting of the cells was done by the hemocytometer.

Furthermore, each supplement was omitted from the low Ca ++ medium and proliferation rate (in triplicate) was measured. The proliferation assay was performed in tissue culture cluster plates (96 flat bottomed wells) (Becton, Dickenson) by incubating 1 $x10^3$ cell/well in 0.2 ml of medium.

Cell proliferation results were measured on day 3,6, and 9 of incubation by the Fluorometric microassay using 4-methylumbelliferyl heptanoate (4-MUH).

Fluorometric values were measured by a Titertec Fluroskan II (Flow Lab. Mechenheim, Germany) as previously described [5]. Student's test was used for statistical evaluation and p values of <0.05 or less were considered significant.

Immunohistochemistry :

Cell suspension of 2nd passage HK was adjusted at $5X10^3$ /ml. A cytospin preparations were made and immunohistochemical labelling was performed with alkaline phosphatase anti-alkaline phosphatase (APAAP) technique as previously described [6]. Briefly, the cytospin preparations were fixed over 30 min. with acetone and were exposed to the primary antibody (30 min) diluted in 0.1 M phosphate buffered saline (PBS) at the concentration listed in table 1. As a secondary antibody anti-mouse IgG (Immunotech. Marseille, France) diluted in 1:100 in PBS was used with the APAAP complex (Dako, Glostrup, Denmark). The incubation steps with the secondary antibody and the APAAP complex were repeated twice to intensify the labelling reaction. Naphthol AS. Bl sodium salt (Sigma, St. Louis, USA) and new fuchsin (Merch, Darmstadt, Germany) were used for visualization. Lastly, counterstaining with Mayer's hemalaun solution (Merck) was done. The slides were mounted with Kaiser's glycerol gelatin (Merck) and examined by light microscopy. Positive staining appears bright pink or red, whereas negative staining acquires the counterstain as faint blue color.

Results :

The data presented in this work provide evidence that HK can be grown in vitro in pure form without the need for serum, substrate modification or feeder cells. Attachment of cells is evident 24 hrs after inoculation and the cells maintained on low Ca ++ level show strong proliferation following attachment and reach confluence in 2 ± 0.1w. The cells are characterized by being polygonal and the cellular boundaries are easily recognized (Fig.1). Higher power examination fails to show any evidence or intercellular bridges (Fig. 2). These cells can be easily passaged and secondary cultures maintained on low Ca++ level showed the same characters of the primary cells, i.e., polygonal in shape, high proliferation rate and absence of intercellular bridges. Cells that are switched to high Ca++ level, show illdefined cellular boundaries, flattening of the cytoplasm and angulation after 24 h (Fig.3). This pro-

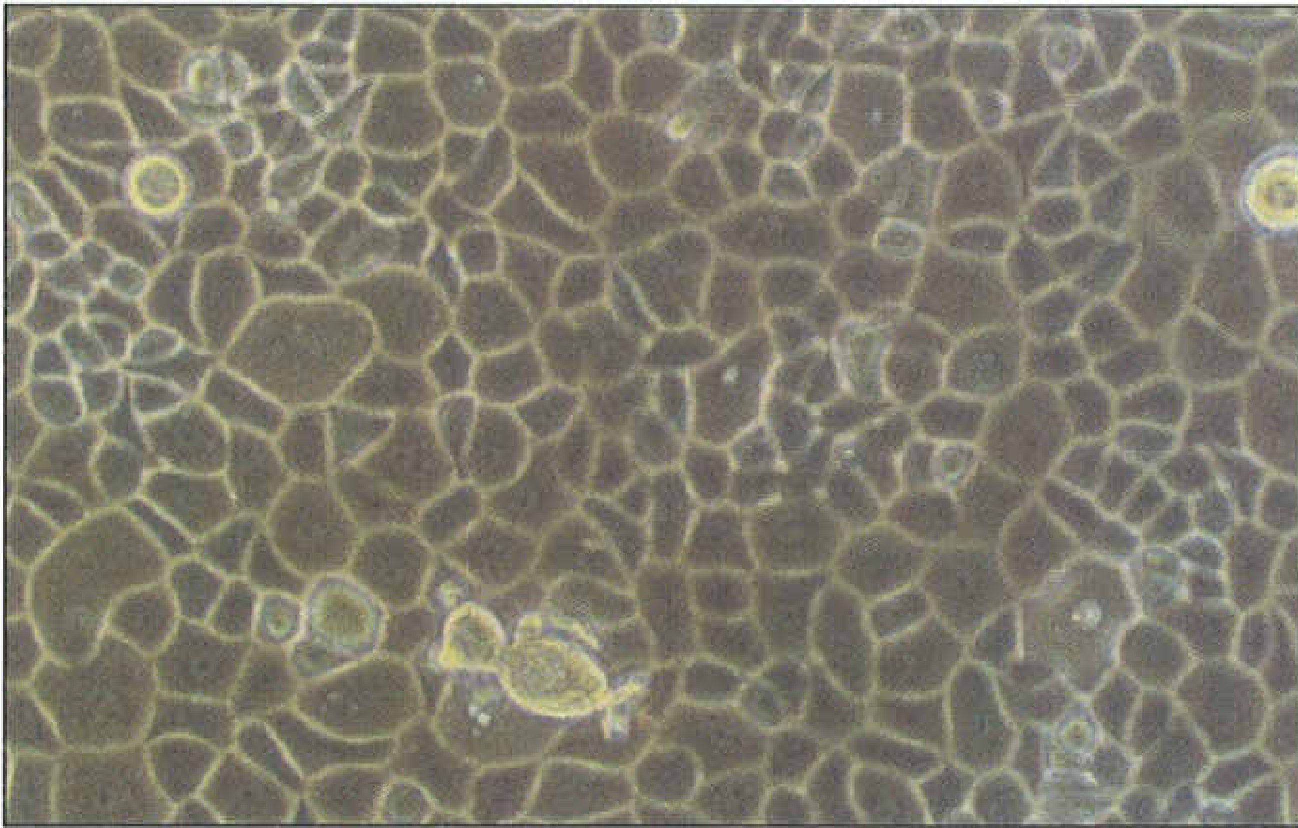

Fig. 1. Sheets of polygonal undifferentiated keratinocytes 7 days after primary inoculation in a low Ca++ keratinocyte growth medium (0.15 mM) (X250).

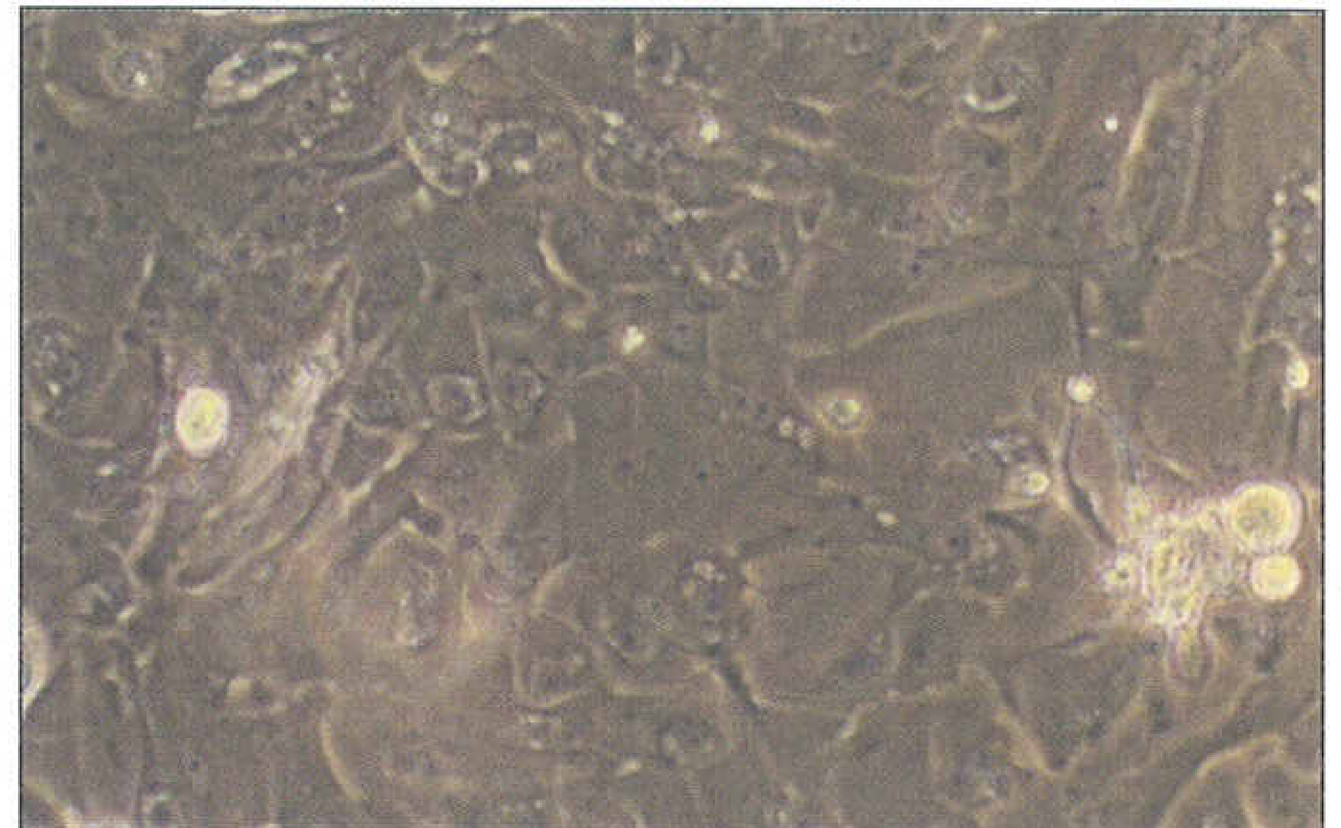

Fig. 3. The same keratinocyte culture 48 h after incubation in a keratinocyte growth medium containing high Ca++ level (1.8 mM). Although the nuclei are still evident, cellular boundaries are less prominent (X250).

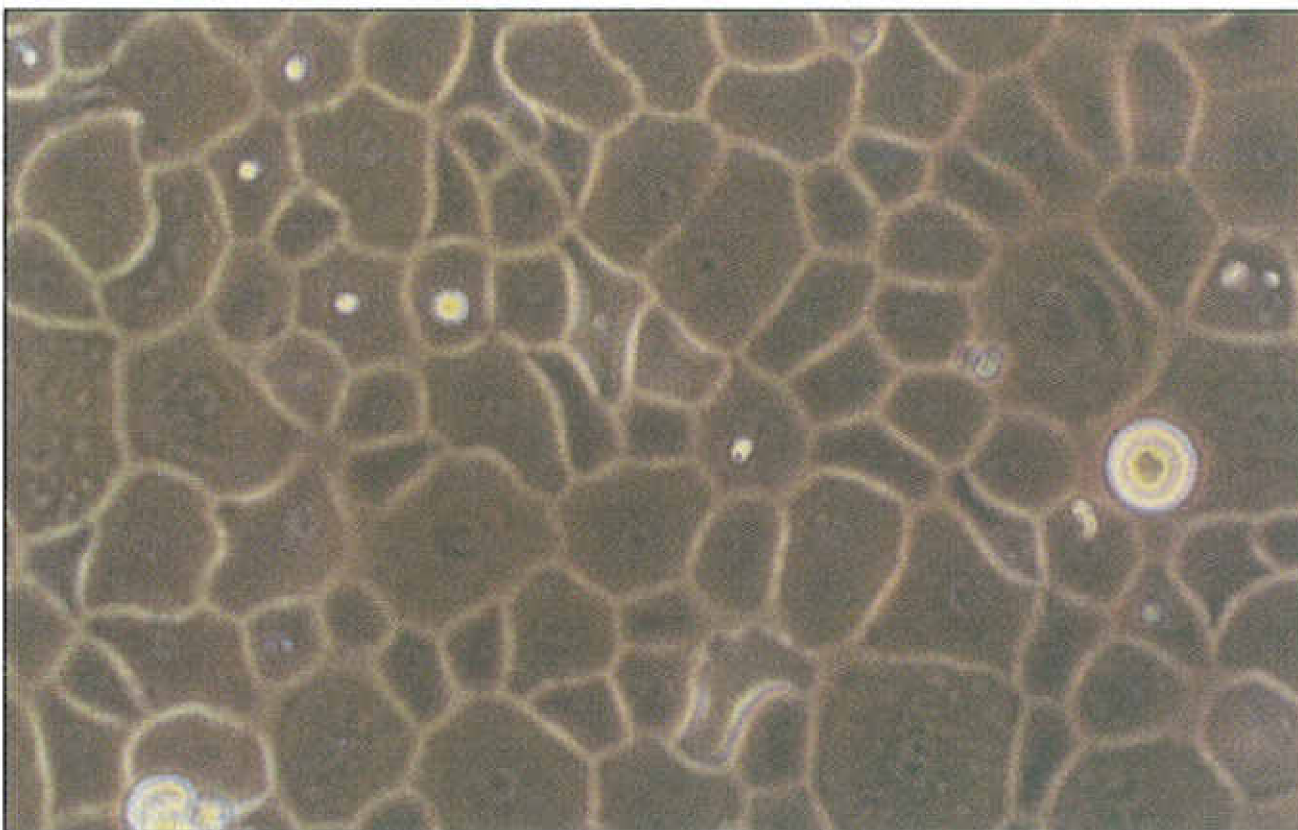

Fig. 2. Higher magnification of primary keratinocyte culture maintained on low Ca++ level. The cellular boundaries with no evidence of intercellular bridges can be easily detected and the nuclei and the promient nucleoli are evident (X400)

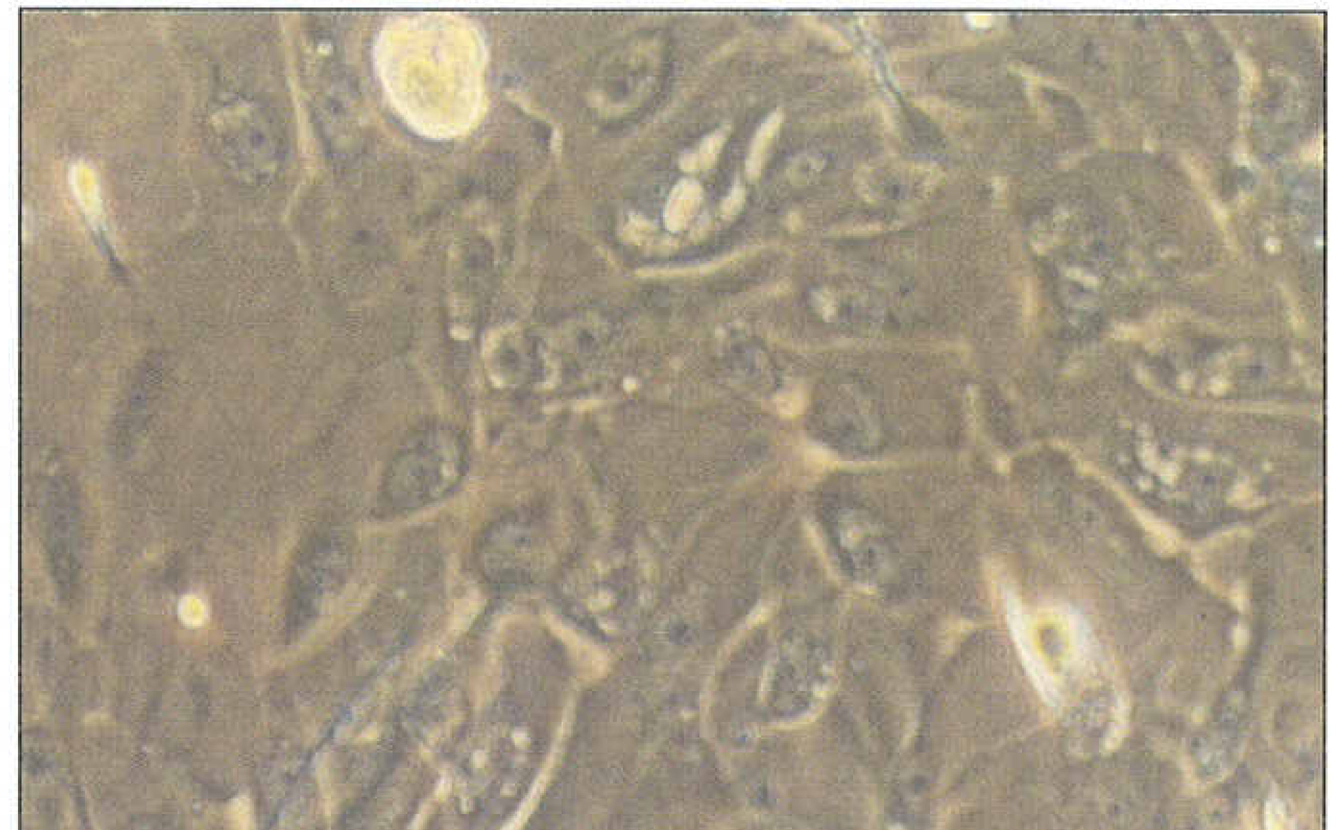

Fig. 4. The same keratinocyte culture 5 days after incubation in a high Ca++ level. The cellular boundaries are ill-defined and refractile granules started to appear in the cytoplasm (X250)

cess continues to several days during which the cells differentiate, show evidence of refractile granules formation and are completely separated from the culture plate after 3-4 w (Fig.4-6).

Proliferation assay :

In general, there is no morphological difference between HK obtained from newborn and adults but the mean cell cycle for the former is 22± 1.5 h whereas it is 25± 0.9 h with the latter ($p<0.05$). Also, differentiated newborn HK separated as a single continuous sheet, whereas adult HK separated either individually or as discontinous sheet. BPE is a strong stimulus for proliferation of HK when the Ca++ level was kept low. Other growth factors also result in stimulation of proliferation but to a lesser extent.

Proliferation studies of cells deprived from BPE showed >50% reduction on day 3,6 and 9 ($p<0.001$) whereas only about 20% reduction of the proliferation in the absence of either EGF, insulin or hydrocortisone on day 6, and 9 ($p<0.05$) (Tab.2, Fig.7).

Immunohistochemistry :

In general no phenotypic differences in cytokeratin expression were detected between newborn and adult keratinocytes. Primary and secondary cultures maintained on low C++ level express CK5, 14 characteristic of basal cells, whereas HK that have been switched to high Ca++ level express cytokeratins of suprabasal cells, namely CK1, 10.

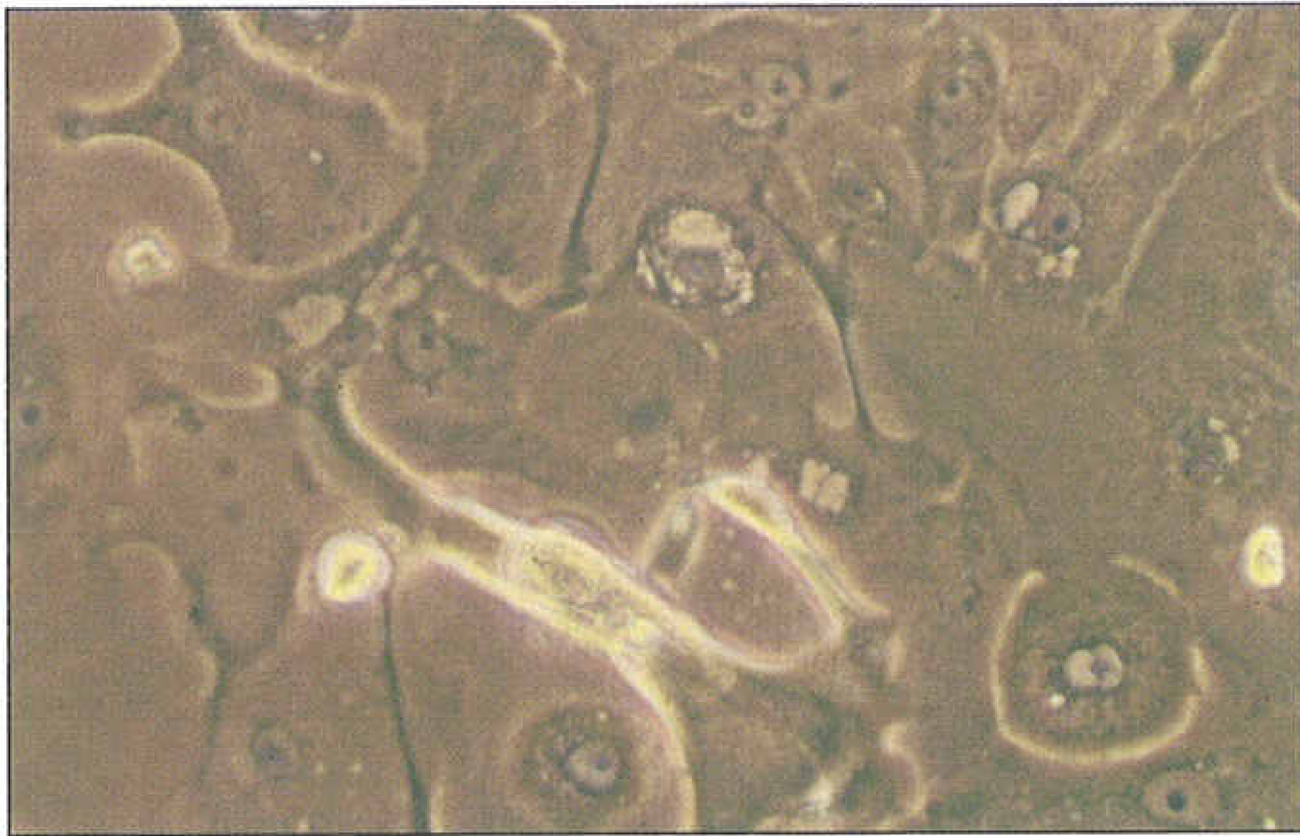

Fig. 5. As the culture gets older (2W) differentiated cells separate from the culture plate and the remaining attached cells become distinct (X250)

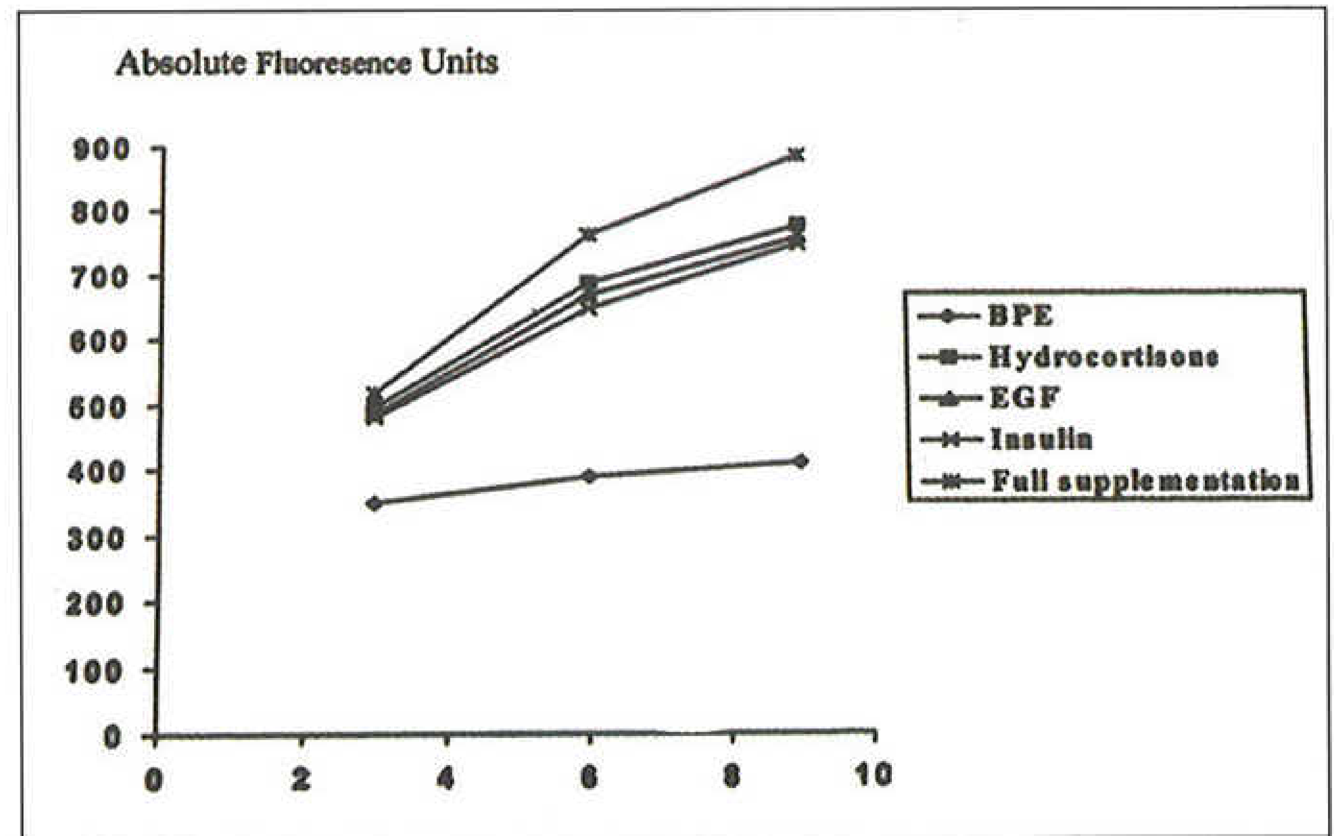

Fig. 7. Proliferation rate of HK expressed as absolute fluorescence units incubated in serum free medium deficient in each supplement. Growth rate of HK is markedly reduced in the absence of BPE.

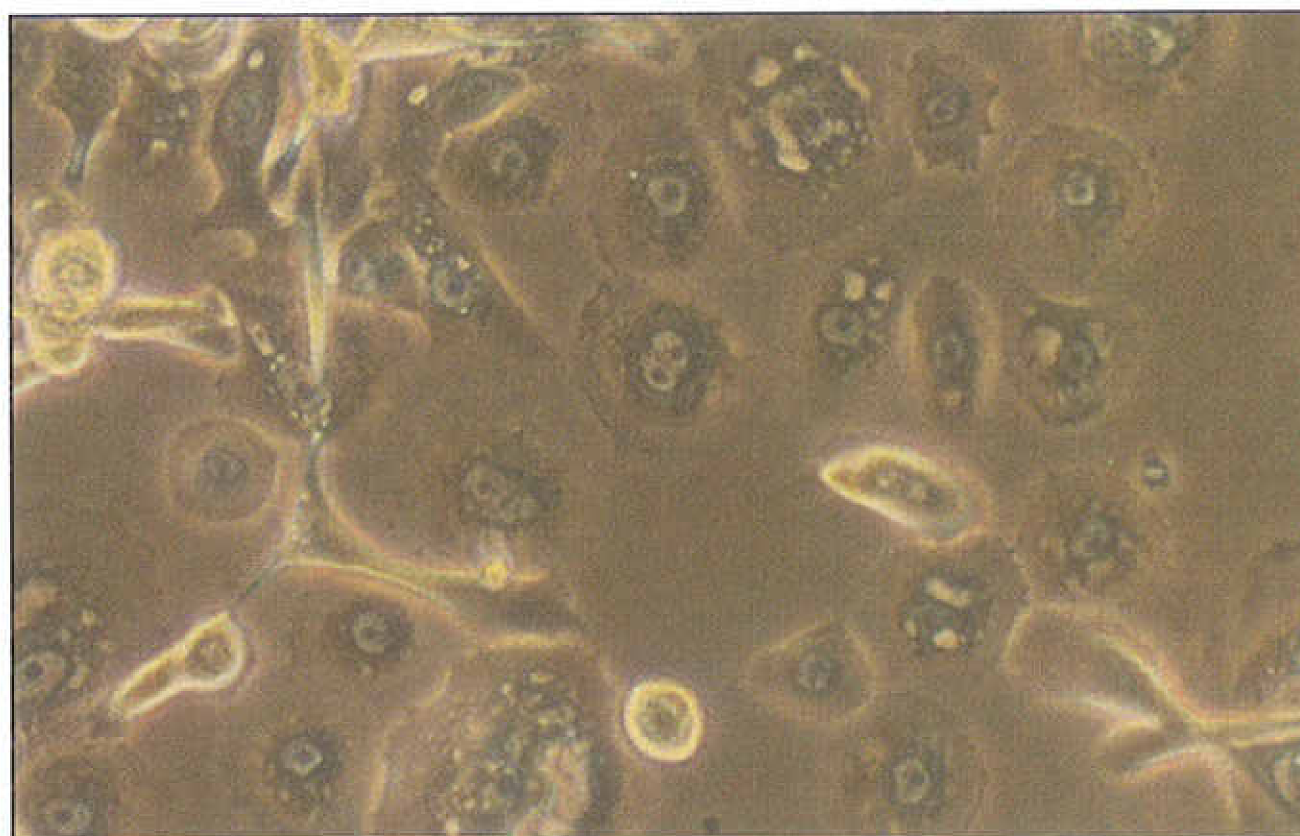

Fig. 6. The same findings with marked refractile granule formation of the cytoplasm (X250)

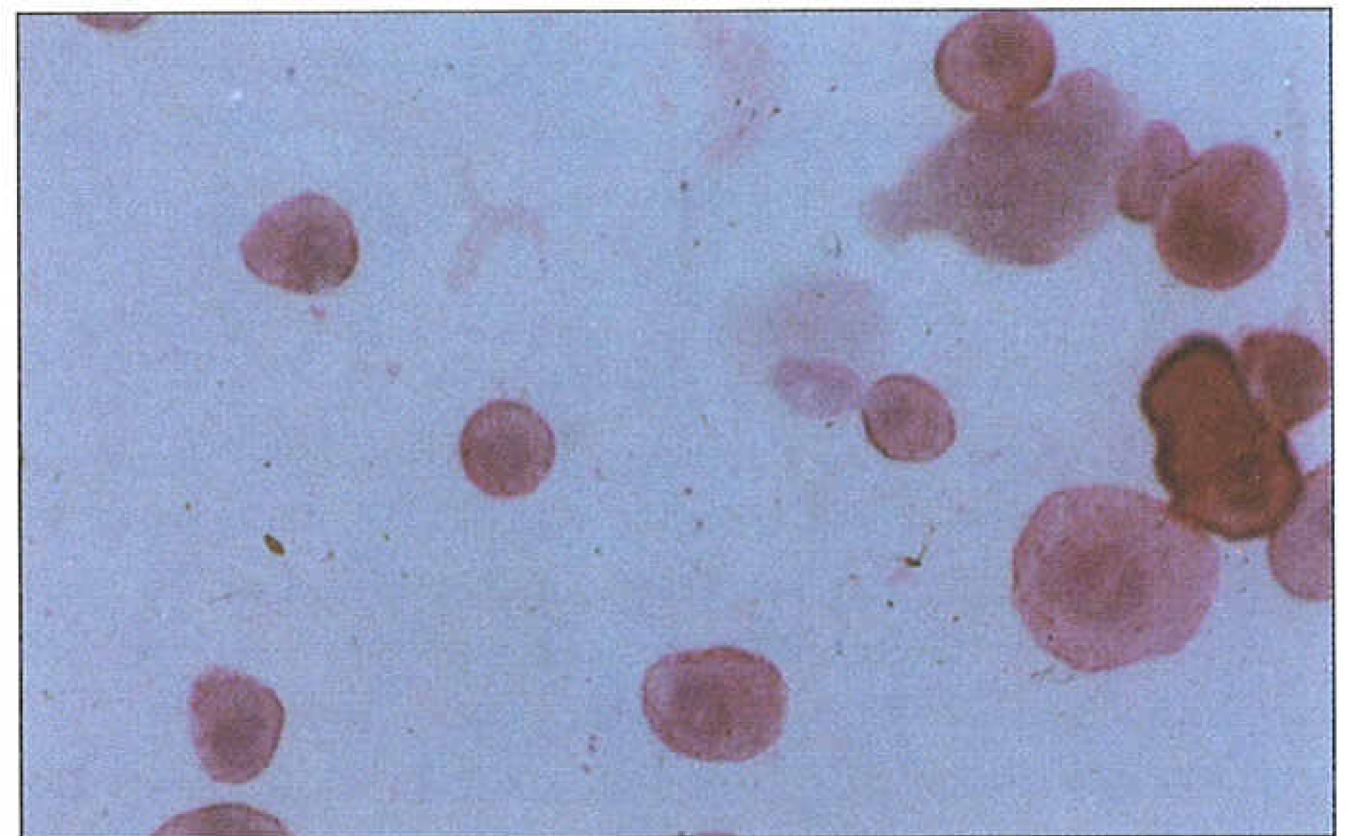

Fig. 8 A cytospin preparation of secondary HK maintained on high Ca++ level stained with CK1 (APAAP complex). All cells showed positive staining but with varying degrees.

Very few (not exceeding 3%) negative cells are observed only in the primary culture maintained on low Ca++ level. These non-keratinocyte cells are not seen in the secondary cultures, whether kept on low or high Ca++ levels. Furthermore, HK maintained on low Ca++ level show uniformal color reflecting the same stage of differentiation, whereas HK maintained on high Ca++ level show variable degrees of positivity ranging from mild redness to strong bright red color denoting various stages of differentiation (Fig.8).

Discussion :

Early studies showed that epidermal HK can be cultivated only in the presence of serum and irradiated mouse transformed fibroblasts (3T3) cells. In this model HK were evident as islands inbetween the supporting mouse fibroblasts [3].

Indeed this model is still in use in investigative work since HK can be easily and rapidly obtained.

Nevertheless HK grown with this method cannot be used in any therapeutic purposes due to the presence of foreign proteins such as fetal calf serum, cholera toxin and foreign cells, such as the mouse transformed irradiated fibroblasts. In this work we could show that keratinocytes can be grown and propagated in vitro without serum, feeder layer or cholera toxin. Furthermore through monitoring of the Ca++ level, we can have cells with the character of either basal or suprabasal

keratinocytes [4,7]. This can be of great help in understanding of disorders of keratinization and in studying several cytokines that may influence keratinocyte differentiation. It has been proven that modified MCDB 153 medium improves HK growth by increasing nutrients that are deficient and by reducing amount of nutrients that are toxic in excess as well as by supplying nutrients that are missing from conventional media [4]. Also previous studies have shown that MCDB 153 medium is not optimal for fibroblast proliferation. Interestingly, low Ca++ level (0.15 mM) is also suboptimal for growth of fibroblasts [8] and has no effect on melanocyte growth [9]. This may provide an explanation of the presence of few dendritic cells in the primary culture, which were absent in secondary culture and in cytospin preparations of our culture system. Therefore, we believe that the modified MCDB with added supplements represents an optimal and selective medium for isolation and propagation of keratinocytes.

HK grown at low Ca++ show several features of basal keratinocytes of the epidermis. They show strong proliferative activity, are polygonal in shape and above all they express cytokeratins 5, and 14 that are specific to the germinative basal cells [10,11].

The only difference between them was the absence of any desmosomal structures, which may be explained by insufficient substrates or enzymes that are necessary for the build up of the desmosomal proteins. Accordingly, the term prokeratinocytes has been proposed to describe these cells in vitro [11]. Previous studies showed that Ca ++ increases intercellular c AMP levels and initiate DNA synthesis, a mechanism that may be responsible for the proliferation of HK [12]. However, in excess, it results in induction of differentiation a process that is characterized by cessation of proliferation, induction of stratification and shedding from the culture plate [13], features that have also been observed in our culture system. We have also confirmed the differentiation process by showing that these cells express cytokeratins characteristic of suprabasal cells, namely CK1 and 10 [10].

BPE has been shown to increase the rate of keratinocyte proliferation. Indeed it is also essential for proliferation of other epidermal cells, e.g., melanocytes and sebocytes [9,14]. In the absence of BPE, marked reduction in HK proliferation occurred (>50%) and therefore it is considered one of the essential supplements of this culture medium. On the other hand, absence of any of the other growth factors, namely EGF, insulin, or hydrocortisone resulted in mild decrease of cell proliferation (ca.20%). At the present time, it is not known exactly what growth factor in the crude BPE is responsible for the marked stimulation of HK in vitro [4].

With the use of the fully supplemented MCDB 153 medium, the growth rate of HK in vitro is very rapid accounting for less than 24h whereas it varies in normal skin from 50-300h [15] and in psoriasis, a hyperproliferative epidermal dermatosis, about 50 h [16].

This renders this model a very efficient method for obtaining pure undifferentiated HK not only for investigative work but also for grafting of extensive burns, chronic leg ulcers, giant melanocytic nevi and correction of hypospadias [17-20]. Interestingly, no morphologic or phenotypic differences were found between newborn and adult HK. Nevertheless, newborn HK, similar to other epidermal cells, showed stronger proliferation rate when compared with adult HK. Indeed, this is an unavoidable obstacle in most cell culture models which should be considered in auto- grafting of old individuals [9,17].

In conclusion, a culture system now exists that permits growth and control of differentiation of normal epidermal HK in defined medium. In addition, this system through change of Ca++ level, can yield either undifferentiated (prokeratinocytes) or differentiated keratinocytes that can be utilized for the study of a variety of medical questions and in therapy.

Table 1.

Antigen	MoAb	Reactivity	Source	Dilution
CK1	K1	suprabasal HK	Enzo	1:4000
CK10	K10	suprabasal HK	Monosan	1:150
PKK2	K5,14,15, 16,17	Basal HK	Labsystem	1:350

Table 2.

Day	BPE	Hydrocortisone	EGF	Insulin	Full Supplemen-tation
3	350 ± 25.1	498 ± 20.3	487 ± 15	480 ± 20.45	17 ± 9.9
6	390 ± 23.2	690 ± 35.7	670 ± 30.2	650 ± 21.1	765 ± 18.2
9	410 ± 17.5	780 ± 27.4	760 ± 26.2	750 ± 15	890 ± 19.7

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