tinal epithelial cells (SLC and ESKI cell lines). The functional insertion of the activated human *Ha-ras* oncogene or polyoma middle T, an activator of the $pp60^{src}$ tyrosine kinase, is associated with the induction and activation of the cancer growth of SLC-44 cells and human colonic cells CaCo-2 in the nude mice. These immortalized and transformed cell lines may thus be useful for studying many aspects of gastric and colonic cancers, including proliferation, differentiation, metastasis and the MDR phenotype. The development of similar immortal cell lines in cystic fibrosis, such as the intestinal CFI-3 cells [5], may also improve our understanding of the biochemical alterations related to genetic defects in man.

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Characterization of human skin keratinocytes immortalized with SV40, HPV, or spontaneously

Petra Boukamp, Dirk Breitkreutz, Pascal Tomakidi, Norbert E. Fusenig

Due to the impressive development in cell culture methodology cell lines could be established from nearly all tissues. However, there exist fewer cell lines of human than of rodent origin. This in as much is remarkable as, for example, normal human keratinocytes are much more easy to culture than normal rodent cells (they can be passaged and thereby increased in number several fold). Nevertheless they have been found to be largely resistant to transformation *in vitro* using either chemical or physical agents [1-4]. The basis for the discrepancy in transformation sensitivity is still unclear. Explanations have been postulated including interspecies differences such as natural life span, degree of inbreeding, or different genetic stability [2, 5].

The first agent described leading to immortalization of human keratinocytes was simian virus 40 (SV40) [6]. This potent DNA tumor virus induced a high number of cytogenetic aberrations [7] which most probably were causally related to the escape from senescence. Similar to SV40, other DNA tumor viruses — human papilloma virus 16 and 18 (HPV) have also proven to immortalize human keratinocytes reproducibly. Thus, a number of cell lines could be established in the meantime in several laboratories using both virus types [8, 9].

A third possibility to establish cell lines, but obviouly a very rare event, is spontaneous immortalization, i.e. escape from senescence without addition of any known transforming agent. To date there exist only two epidermal keratinocyte lines, one derived from a culture of foreskin keratinocytes (NM1 [10]) while the other, HaCaT, originated from trunk skin [11]. The actual cause of transformation is still elusive. However, there are indications that elevated temperature (one of the conditions of the initial culture phase of the HaCaT

P. Boukamp, D. Breitkreutz, P. Tomakidi, N.E. Fusenig : German Cancer Research Center, IM Neuenheimerfeld 280, Heidelberg, Germany.

cells) increases the risk for chromosomal aberrations. Furthermore, Sugawara and coworkers [12] recently proposed so called senescence genes which are lost in immortalized cells. They could show that after introduction of a chromosome, carrying the particular chromosome region, these cells regained senescence. Some of these genes are obviously located on chromosome 1 and also on chromosomes 4 and 9. This is of particular interest since the latter two chromosomes were involved in the first and further stable cytogenetic rearrangements seen in the HaCaT cells. Thus, it seems reasonable to speculate that immortalization of the HaCaT cells has been caused by loss or alterations in the senescence gene(s).

The most intriguing finding of the early investigations was that foreskin keratinocytes, immortalized with SV40 had lost their differentiation capacity. From this it was concluded that a block in differentiation might be an early and obligatory step for immortalization of human keratinocytes [13]. These observations were based on alterations examined under *in vitro* conditions. In culture, however, even normal keratinocytes undergo incomplete or aberrant differentiation due to the lack of instructive or permissive mesenchymal factors, present *in vivo* [14]. This became particularly evident when we analyzed the differentiation potential of a cell line (HaSV) obtained after transfection of trunk skin keratinocytes with origin defective SV40 DNA. *In vitro* these cells did neither stratify nor form horn-squames and, compared to normal keratinocytes, they exhibited a largely abnormal keratin profile. Epidermal keratins K5, K14 (basal) and K1, K10 (differentiation specific) were only faintly expressed at best while so called simple epithelia-type keratins, characteristic for nonstratifying simple epithelia [15] were predominant.

In order to distinguish if the altered differentiation reflected a stable trait of the HaSV cells or if differentiation could still be modulated by appropriate stimuli, the cells were analyzed under *in vivo* conditions.

In general *in vivo* growth and differentiation behavior is difficult to determine since most of the cell lines are non-tumorigenic. Moreover, the generally used injection site (subcutaneously) does not necessarily represent the optimal location to test these parameters. We have developed a transplantation assay where the cells are precultivated on a collagen gel (type I) *in vitro* and then transplanted onto the muscle fascia lata of nude mice as intact cultures [16]. In this configuration the epithelium is separated from the mouse mesenchyme by a solid collagen matrix, a situation comparable to the skin. Similarly, the orientation and flow of nutrients in this system resembles more closely that ot the epidermis *in situ*. Under these conditions normal keratinocytes form a well stratified and differentiated epidermis within 6 days after transplantation [14]. Moreover, since even minor inherent variations between keratinocytes from different body sites became apparent in this assay it should also be sensitive enough to detect alterations caused or accompanied by immortalization.

When the SV40 immortalized HaSV cells were transplanted onto nude mice, these cells still formed a multilayered epithelium with the typical epidermal strata. Moreover, the analysis of the keratin profile showed that the « simple epithelia-type » keratins were largely down-regulated or even completely suppressed while the epidermal keratins (K5 and K14) became predominant and also one of the differentiation specific keratins (K10) was induced. On the other hand, according to this transplantation assay, another SV40 immortalized cell line, SVK-14 [17], seemed to have irreversibly lost its differentiation potential. Thus, the comparison of these two cell lines clearly demonstrated that loss of differentiation may be associated to immortalization but is obviously not a prerequisite.

Further, HPK-I and HPK-II, two cell lines obtained after transfection of foreskin keratinocytes with HPV 16 DNA, were analysed in more detail [18]. Compared to the HaSV cells they resembled much more normal keratinocytes in culture as indicated by morphology, ability to stratify and their *in vitro* keratin profile. The epithelium formed after transplantation of both cell lines was well stratified with a distinct stratum granulosum and massive stratum corneum. The tissue morphology largely resembled a mucosa-type epithelium, perhaps reflecting the origin (foreskin) of the cells.

The third group of cells analyzed under comparable conditions were the spontaneously immortalized cell lines HaCaT and NM1. The HaCaT cells are in culture for more than 6 years (> 300 passages) and despite a number of additional cytogenetic changes they remained non-tumorigenic. Similar to the HPK cell lines also the HaCaT cells largely resembled normal keratinocytes *in vitro*. During passaging they gradually lost the ability to stratify or form horn squames *in vitro* but they remained sensitive to differentiation inducing stimuli such as high cell density or retinoic acid depletion [19, 20]. Similarly, under *in vivo* conditions (in transplants) these cells (up to passages < 240) were able to form well differentiated epidermis-like epithelia showing strong expression and regular localization of the typical epidermal diffentiation markers. With cells of higher passage levels, only dysplastic and morphologically undifferentiated epithelia were observed. NM1 cells, on the other hand, were not able to stratify in our transplantation assay. Nevertheless, despite a few-layered and largely unorganized epithelium, these cells expressed the differentiation specific keratins K1 and K10.

In conclusion we found (i) a wide spectrum of differentiation and (ii) a high variability among the members of the respective modes of immortalization when comparing the six different keratinocyte lines. These findings strongly suggest that loss of differentiation is *not* an initial event to overcome senescence. Thus, differences in the differentiation potential might be rather attributed to *in vitro* selection accompanying the early culture phase than to causal events of the immortalization process ■

Résumé

Caractérisation des kératinocytes immortalisés par les virus SV40, HPV *(human papillomavirus)* ou spontanément

Les propriétés et caractéristiques de différenciation de six lignées établies de kératinocytes en culture ont été comparées *ex vivo* et *in vivo* chez la souris *nude*. Les lignées HaSV, SVK-14 ont été obtenues par infection avec le virus SV40, HPK-I et HPK-II l'ont été par transfection avec l'ADN du virus HPV-16, alors que les deux lignées HaCaT et NMI se sont établies spontanément, peut-être par perte de gènes de sénescence. *Ex vivo*, ces cellules ont variablement été différenciées, avec, notamment, une diminution de la synthèse des kératines épidermiques K5 et K15 et une synthèse abondante des kératines caractéristiques des épithéliums simples, incapables de former des strates multiples. Afin de distinguer entre une perte définitive du potentiel de différenciation lié à l'immortalisation et l'absence, en culture, des signaux indispensables à l'expression du programme de différenciation, un système d'étude chez la souris *nude* a été développé. Il s'agit de transplanter des cellules précultivées sur collagène de type I au niveau du muscle fascia lata de souris, sous la forme d'une culture intacte dans laquelle les cellules épithéliales sont, comme au niveau de la peau, séparées du mésenchyme par une matrice de collagène. Dans ces conditions, certaines des lignées subissent une différenciation, mimant plus ou moins complètement la différenciation kératinocytaire normale alors que d'autres restent indifférenciées. Il apparaît ainsi que les anomalies de différenciation ne sont pas une conséquence inéluctable du processus d'immortalisation, mais bien plutôt le résultat d'événements surajoutés au cours des multiples passages *ex vivo*.

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