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Comparative analysis of proliferative activity of oral and epidermal keratinocytes under the influence of epidermal growth factor

Jovan Cabunac [1,*], Sanja Tanasković Stanković [2], Zoran Milosavljević [2], Irena Tanasković [2] and Tatjana Kanjevac [1]

[1] Integrated Academic Studies of Dentistry, Faculty of Medical Sciences University of Kragujevac, Serbia
[2] Histology and embryology Institute, Faculty of Medical Sciences University of Kragujevac, Serbia

Abstract:

Introduction and aim: Keratinocytes are the main cells of the stratified-squamous epithelium that covers oral cavity and skin. Keratinocytes of the oral cavity are, unlike epidermal, under the constant influence of EGF from saliva which increases their mitotic index and accelerates wound healing. The purpose of our research was to compare the proliferation rate of these two types of cells in "*in vitro"* conditions with identical cultivation media supplemented with EGF.
Material and methods: Biopsy material from three samples of both, oral mucosa and of the foreskin, were mechanically and enzymatically treated for primary isolation of keratinocytes. Identical number of cells from both samples is cultivated in culture vessels while adding nutrient medium supplemented with EGF. After 96 hours, the total number of keratinocytes was assessed in order to quantify the influence of EGF.
Results: Primary isolation of epidermal cells provided 2.5 times higher yield than the oral epithelium, but viability of keratinocytes was lower by approximately 25%. Very low and high concentrations of EGF have weak mitotic effect on cells, whereas 5ng/L of this hormone gave the highest yield of both oral and epidermal keratinocytes. The yield of oral keratinocytes was by around 16% higher. The absence of this growth factor leads to a more rapid aging and growth arrest of the cultivated cells.
Conclusion: EGF is necessary for proliferation and differentiation of keratinocytes. Oral keratinocytes have a higher proliferative capacity than epidermal ones when cultivated under the same conditions.

Key words: keratinocytes, EGF, oral cavity, epidermis, proliferation.

Introduction

The stratified-squamous epithelium is the most widespread epithelium in the human body. It can be found with keratosis in the skin epidermis or without keratosis in the oral mucosa. The main cells of both types of epithelium are keratinocytes which proliferate to renew the basal layer epithelium. Migration of the proliferated cells covers the defects and/or leads to wound healing [1]. Oral keratinocytes have twice shorter life span than the epidermal, mainly because their proliferation is boosted by constant influx of epidermal growth factor (EGF) which is an integral part of secretion of the salivary glands [2]. EGF was discovered and isolated in 1962, later research showed that this single-chain polypeptide is linked to its receptor (EGF-R) on the membrane of keratinocytes causing, through a series of dependent products, a stronger expression of certain genes and a higher mitotic index in these cells. In greater concentrations, it increases the motility and migratory capacity of the target cells [3]. This hormone is also detected in small quantities in oesophageal, gastric and mammary glands, urine and serum [4, 5]. The presence of EGF in the serum is important because of its influence on the proliferation and differentiation of the epidermal keratinocyte, since only a small quantity is required for mitotic activity in the basal layer and for keratinization [6, 7, 8]. The average concentration of this growth factor in the serum of healthy individuals is 0.7ng/ml and in the saliva about 10 times more (7ng/ml) [5]. For this reason, defects on the stratified squamous epithelium without keratinization in the oral cavity heal considerably faster than wounds on the skin, that is, epidermis [9, 10, 11]. The purpose of our research is to discover any differences in the proliferation rate between oral and epidermal keratinocytes when cultivated in "*in vitro"* conditions under identical concentrations of EGF.

**Corresponding author: Jovan Cabunac*
Email address: drjcabunac@yahoo.com
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We also aim to demonstrate the migratory effect of this hormone in high concentrations.

Material and methods

In the course of this research, biopsy material of healthy oral mucosa obtained after dental intervention (age of donors 23-63 years) and samples of human skin from healthy foreskin obtained after circumcision (age of the donors 18-33 years) was used. All the procedures were in accordance with the ethical standards and Helsinki Declaration of 1975, as revised in 2008.

Our research includes three samples from each group of tissue. Samples were then treated with povidone iodine solution in order to eliminate microbiological contamination. Samples were mechanically trimmed with scissors in order to remove the excess of connective tissue of the lamina propria of the mucosa and dermal connective tissue, after which they were treated with the solution of Dispase II enzyme (5mg/ml) (Gibco, USA) and antibiotic/antimycotic at +4ºC over night. After digestion, the complete epithelium was carefully separated by tweezers from the underlying tissue and put into trypsin-EDTA solution at 37°C with mild stirring to obtain keratinocyte suspension. Excessive digestion was blocked after 30 minutes by adding calf serum. After rinsing off and repeated centrifugation, cell viability was assessed with acridine-orange/ethidium-bromide. Individual keratinocytes of the oral mucosa and human skin were then cultivated in culture vessels where nutritive medium was added for keratinocytes growth.

The basis of the medium was mixture of DMEM and Ham F12 medium (3:1) with addition of insulin 5µg/L, transferrin 5µg/L, hydrocortisone 0,4 µg/L, adenine 24,3 µg/L and epidermal growth factor in four different concentrations (0, 2, 5 and 20 ng/L) (Invitrogen, USA). The medium was changed on alternate days.

Second passage of keratinocytes was performed in order to obtain the same number of cells for each tissue sample (10,000/cm^2). Cells were then cultivated in 6 different cultivation fields originally coated with poly-L-lysine and left 24 hours to completely attach to the bottom of the vessel. The total number of cells obtained by trypsinization after 72-hours experiment was determined in each cultivation field by cell counting chamber. Results were statistically processed using the commercial SPSS software 2.0 and statistical significance was determined by Student's t-Test. P values below 0.05 were considered significant. All procedures were performed in triplicate.

Results and Discussion

Enzyme dissociation of all samples of the biopsy material and trypsinization of epithelial cells produced suspension of individual keratinocytes, but cell yield notably varied in the samples of oral mucosa and foreskin. We found that in 1cm^2 of surface over 2.5 times more cells were produced by primary isolation of keratinocytes of the epidermis than by oral epithelium. Viability was assessed by counting cells under the fluorescent microscope, and was for the primary isolated cells of the oral epithelium 84±6 % while 62±12% for the epidermis.

When cultivating the second passage (10,000 cells/cm^2), only less than 1% of cells did not adhere tightly within 24 hours to the lining in both cases (oral epithelium and epidermis) which did not influence the final results of the experiment. Our results showed that the EGF influenced cell yield, that is, proliferation of the cultivated cells, whereby a concentration of 5 ng/L increased the mitotic index most significantly (Fig 1.).

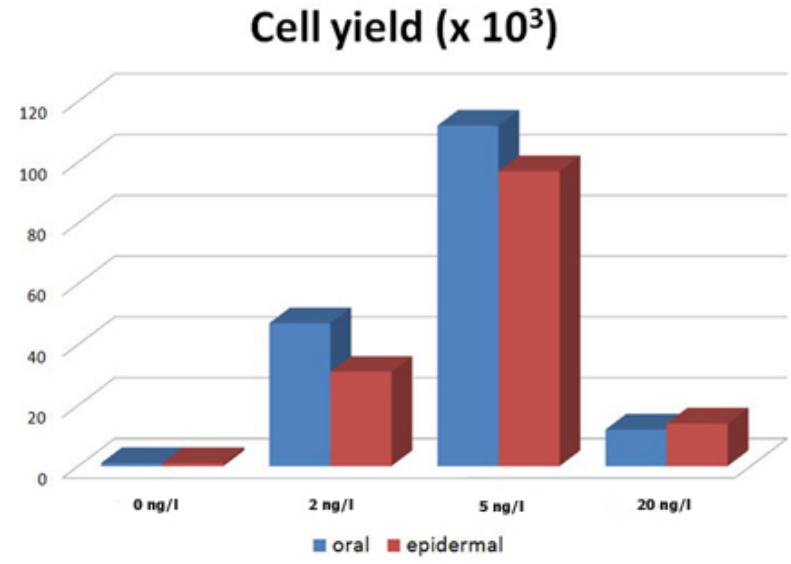

Figure 1. Cell yield of oral and epidermal keratinocytes cultivated at various concentra-tion of EGF (in ng/L)

The control group of cells (EGF 0 ng/L) did not show any signs of proliferation. The concentration of 2ng/L EGF in the medium weakly increased the mitotic index of keratinocytes. Oral vs epidermal keratinocytes cell yield at the end of the experiment in this case was 47 x 10^3 vs 31 x 10^3, while at the concentration of 20ng/L ng EGF we noted an increased migratory, but weak proliferative effect (Fig. 2).

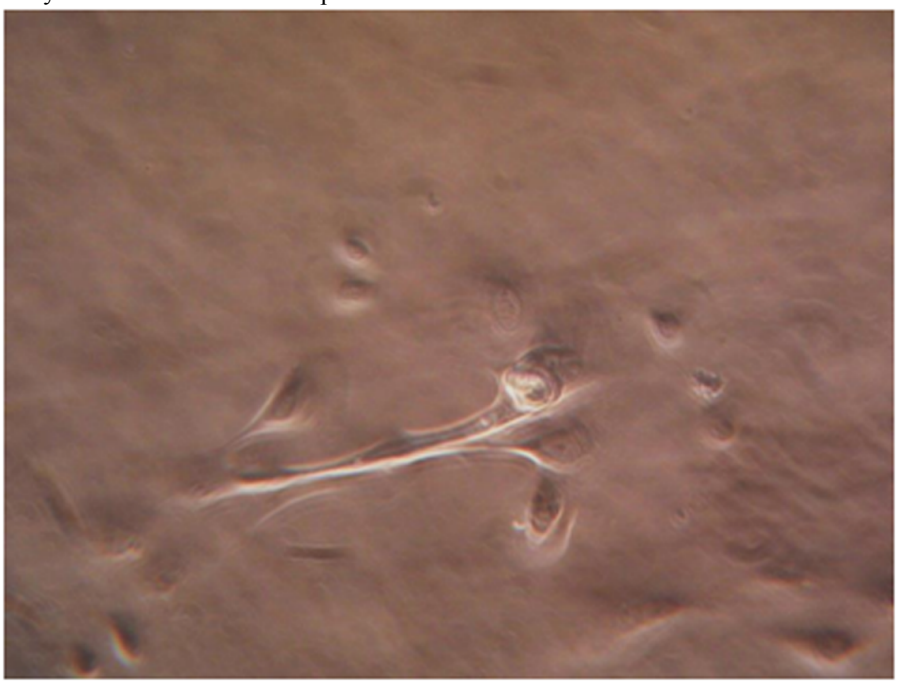

Figure 2. Strong migratory and low proliferative effect of EGF (20 ng/L) on keratinocytes,(mag X 400)

After 96 hours, during which all keratinocytes were cultivated under identical conditions, the highest yield of cells from oral epithelium was achieved at a concentration of EGF of 5ng/L (112±17 x 10^3) (Fig. 3), while the number of epidermal keratinocytes under the same conditions was 97±13 x 10^3 (Fig. 4).

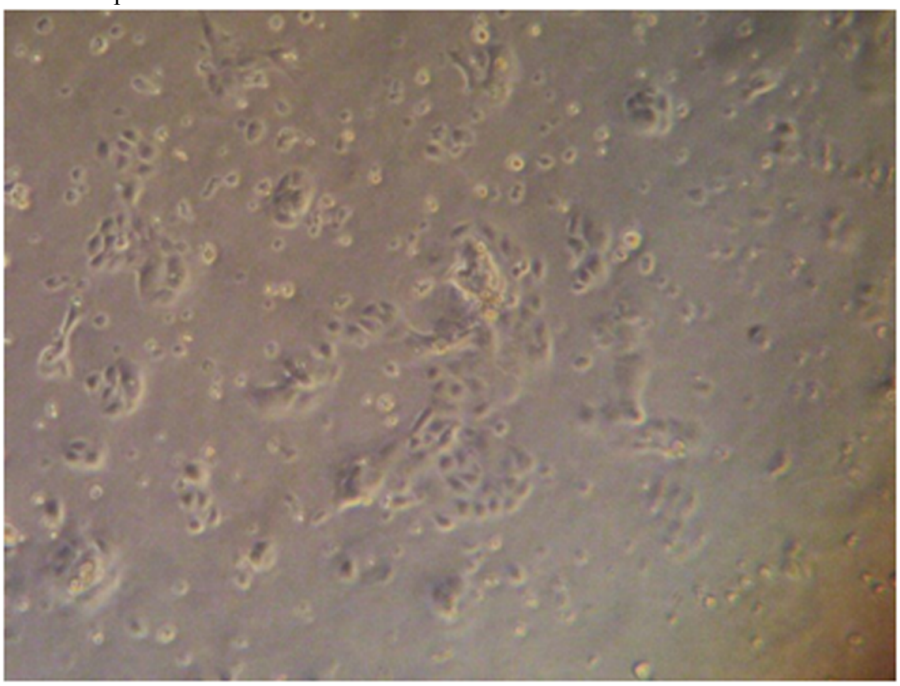

Figure 3. Oral keratinocytes cultivated with 5 ng/L of EGF (mag X 100)

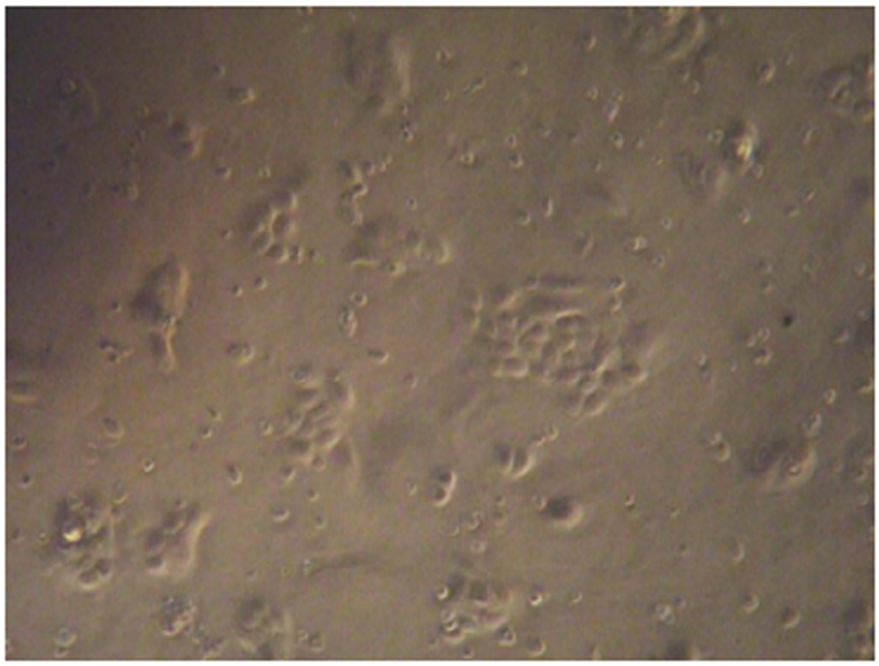

Figure 4. Epidermal keratinocytes cultivated with 5 ng/L of EGF (mag X 100)

Our results have shown that the yield of oral keratinocytes was by 15.5% higher than the yield of epidermal keratinocytes under the same cultivation conditions. Statistical analysis showed statistical significance ($p<0,05$) between the yields of the two types of keratinocytes when cultivated with 5ng/L of EGF. Only in one case, with the donor of oral mucosa aged 63 years, a significant departure in the number of keratinocytes at the end of the experiment was seen when compared to the other values from the same group (EGF 5ng/L=67 x 10^3 cells). Therefore the mentioned result was excluded from the final results.

Keratinocytes are the main cells of the stratified-squamous epithelium which is present, without keratinization in the oral mucosa and with keratinization in the skin e.g. epidermis. The most important factor for proliferation and differentiation of these cells is EGF, growth factor which can be detected both in the saliva and the serum, and in other secretions and body fluids [2, 3, 5, 6]. A great number of researchers have examined the impact of EGF on different cells and tissues in "*in vivo*" and "*in vitro*" conditions, so that plenty of information prove proliferative and migratory effect of this hormone on target cells [12-19]. However, the literature offers little information about the comparative effects of different concentrations of EGF on normal oral and epidermal keratinocytes. Castro-Muñozledo et al., Zare et al. and Xiao et al. found that EGF at low concentrations (from 2ng/L to 10 ng/L) accelerates the proliferation of keratinocytes of oral and epidermal origin [13, 14, 18]. On the other hand, Dickhuth et al. and Turabelidze et al. showed that EGF at the concentration of 5 ng/L gives approximately 20% higher yield of oral than epidermal keratinocytes, although they are cultivated under the same conditions [19, 20]. Our results confirm the findings of the above-mentioned studies, but during our research oral keratinocytes proliferated by 15.5% faster than the epidermal keratinocytes under the influence of 5ng/L EGF. Unfortunately, in the available literature we could not find sufficient information about the comparative analysis of the proliferative capacity of these two types of keratinocytes in case of relatively low (2ng/L) or high concentration of EGF (20ng/L). Our results support the finding presented by Gibbs et al. with regard to the speed of proliferation of keratinocytes under the influence of low concentration of the tested growth factor [21]. In their study they found that the epidermal keratinocytes increase their number during 7 days of cultivation nearly 45 times, which basically corresponds to our findings, if we re-calculate our results to that number of days. With regard to the keratinocytes of the oral epithelium, the previous studies showed considerable discrepancies. We could agree, when speaking about the cultivation of these cells under the influence of 5ng/L EGF, with the finding of Zhou et al. who state in their results that within 7 days of cultivation the number of epidermal cells of oral origin was increased by 55 times [11]. In the case of cultivation of keratinocytes at high doses of EGF (in our study 20ng/L), our results proved the opinion of Seeger et al. who stated in their study a significant migratory effect, but a low proliferative effect of this growth factor when administered at high doses [22]. In our study, migratory rather than proliferative effect of high doses of EGF, was showed by single cell tracking at the beginning of the cultivation (Fig 2) and significantly lower cell yield at the end of the experiment, but in order to further analyse and quantify this migratory effect,

scratch test or snail gene detections should be performed.

Conclusion

Based on the results from our study, we can conclude that oral keratinocytes show a stronger proliferative response than the epidermal keratinocytes (approx. 16%) when cultivated under the identical conditions. Although both cell types belong to the same type of epithelium (stratified squamous), it is obvious that some other factors, beside EGF stimulation are important for such behaviour. Further investigation are requested to clarify these findings, probably aimed to EGF receptor quantification and degree of activity as well as examination of the intrinsic pathways of the tyrosine-kinase in both cell types.

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