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Keratinocyte Proliferation in Aged Rat Skin by High Voltage Pulsed Current Stimulation

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The purpose of this study was to determine the effect of high voltage pulsed current (HVPC) stimulation on proliferative activities of basal keratinocytes by measured nucleolar organizer region (NOR) expression and thickness of spinous layer in aged rat skin. Fifty-one weeks old twelve male Sprague-Dawley rats (300~350 g) were divided into control and HVPC stimulation groups. Each animal's hair on the back were removed. The HVPC stimulation group received an negative monophasic twin peak pulsed current stimulation with 50 V, while the control group was given the same treatment without electricity. The rats were sacrificed after 3 weeks. The biopsy specimens were fixed in formalin, embedded in paraffin and stained with hematoxyline-eosin and silver nitrate. The thickness of basal to granular layer of the epidermis were measured using a light microscope and computerized image analysis system. The number of argyrophilic nucleolar organizer region (AgNOR) were counted using a light microscope and computerized image analysis system and calculated as the mean number of AgNOR per nucleus in the basal keratinocyte. By using a Student's *t*-test, an increase in the thickness of basal-spinous layer ($P<0.001$) of epidermis can be observed in HVPC stimulation rats as compared with the control rats, whereas the thickness of the granular layer is not affected. A Student's *t*-test showed a significantly higher mean NOR number per nucleus of the basal keratinocyte in the HVPC stimulation rats than control rats ($P<0.001$). There was significantly positive correlation between the NOR number and the thickness of basal-spinous layer ($r=0.80$, $P<0.05$). These results suggest that the HVPC stimulation may increase the thickness of spinous layer in the epidermis due to increased proliferative activities of basal keratinocytes in epidermis in aged rat skin.

Key Words: Aged rat, Electrical stimulation, Epidermis, Keratinocyte, Proliferation

INTRODUCTION

In the last 30 years, there has been an increasing interest to investigate the effect of endogenous and exogenous electrical signals for biologic tissue growth and repair. A variety types of electrical signals have been used for soft tissue and bone healing. High voltage pulsed current (HVPC) is a type of exogenous electrical signal characterized by twin peak monophasic pulses, delivered at high voltage but low amperage because each pulse durations are extremely short followed long interpulse intervals. HVPC have been used in several countries for medical purposes for many years. In most cases, HVPC stimulation as an electrotherapeutic modality are applied to delayed or non-healed wound. It has been reported to be effective in treating decubitus and diabetic ulcers as well as increasing the speed of epithelialzation, both in experimental animals (Brown et al., 1988) and in humans (Kloth et al., 1988; Feedar et al., 1991; Mulder, 1991; Fitzgerald et al., 1993).

Human epidermis undergoes morphological, electrophysiological, and functional alterations during aging. Epidermis that ages chronologically, is characterized by a variety of morphological changes including a decrease in the epider-

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mal thickness (Sans et al., 1993; Lock-Andersen et al., 1997; Lavker, 1979), flatten the dermo-epidermal junction, decrease the area of contact surface between epidermis and dermis by 35% (Sans et al., 1993), and a lost the intradermal villous cytoplasmic projections of basal keratinocytes (Lavker, 1979; Lavker et al., 1987). Electrophysiologically, the resistance of the epidermis increased due to the decrease in the water content of the stratum corneum with aging (Ngawhirunpat et al., 2002). Functional changes is characterized by decreased mitotic activity, increased the cell cycle duration, and delayed migration time from the basal layer to the stratum corneum by 50% (Engelke et al., 1997; Bauer et al., 1980; Groveet al., 1983). These changes explain the chronic degenerative changes including wrinkles, dryness, scaly, mottling and blotching formation in aged skin. These changes account for the decrease of protection role, immunological defense, healing ability, easy fragility to shear stress and decreased permeation of substances through the skin in aged skin.

The epidermis is a dynamic structure, the integrity of which depends on a constant mitosis of new keratinocytes in the basal layer and their outward migration and differentiation. It is known that various exogenous electrical signals play an important role in proliferation activity of keratinocytes *in vitro* (Hinsenkamp et al., 1997; Szabo et al., 2001; Manni et al., 2002; Manni et al., 2004). However, the effect of HVPC on the proliferative activity of keratinocytes *in vivo* have not been demonstrated.

Our work concerned the effect of HVPC on proliferative activity of basal keratinocytes in aged rat skin. In order to evaluate the possible role of keratinocytes in producing the biological effects of HVPC, we have conducted an *in vivo* study with rat. The purpose of our study was to investigate the effect of HVPC on proliferative activities of basal keratinocytes by measuring the nucleolar organizer region (NOR) expression in the basal keratinocytes and thickness of spinous layer in aged rat skin.

MATERIALS AND METHODS

1. Animals

Fifty-one weeks old twelve male Sprague-Dawley rats (Daehan Biolink Co., Ltd., Korea) weighing (300~350 g) were used. The rats were kept under clean conventional conditions, which they were fed standard rat chow (Samyang Feedstuff, Samyang Co. Ltd., Korea). They had access to tap water ad libitum, and were kept under a 12 h light/dark cycle at a constant temperature of 24±2℃.

2. Electrical Stimulation

The rats were placed in a clear plastic restrainer and rested comfortably. The back skin of the rat were covered with saline-soaked gauze pads (2×2 cm), and the electrode was placed on the gauze and secured in a restrainer. The saline saturated dispersive pad electrode were placed on the rat's abdomen and secures with pads. Electric line cords were inserted through the window in the restrainer. The rat of the HVPC stimulation group were received electrical stimulation with a current intensity of 30~50 V, pulse duration 140 μs, at 120 pps through the electrodes for a duration of 30 minutes using a monophasic twin peak high voltage pulsed current stimulator (EGS® 100 SL, Electro-Med Health Industries, FL, USA). Control rats were given a sham treatment without electricity.

3. Tissue Sampling and Histochemical Staining

Following tissue sampling, the rats were sacrificed by ether anesthesia. A 10×10 mm sample of the back skin was excised and fixed in 10% phosphate buffered formalin. The tissue sample included subcutaneous tissue and musculature. The fixed tissue sections were dehydrated by ascending graded alcohol series, cleared with xylene using an automatic tissue processor (Citadel 1000, Shandon, Life Sciences International Ltd., UK). The tissue sample was embedded in paraffin and cut into 4 μm thick serial sections using a rotary microtome (Rotary Microtome HM 340E, Microm Laborgeräte GmbH, Robert-Bosch-Strasse 49, D-6909 Walldorf, Germany). For each rat tissue sample, serial sections were stained with hematoxyline-eosin and colloidal silver.

4. Quantitation of Epidermal Thickness and AgNORs

Quantitation of epidermal thickness and argyrophilic nucleolar organizer region (AgNORs) were performed using a computerized image analysis. For video image analysis, a light microscope (Olympus BX 50, Olympus Optical Co., Ltd., Tokyo, Japan) was linked to a CCD camera (IK-642K Toshiba CCD color camera, Toshiba Co., Tokyo, Japan), and an image processing and analysis system (Image-Pro® Plus, Media Cybernetics, Inc., MD, USA). The software

used in this system was a WIN98, along with a compute rized image analysis software Image-Pro® Plus (ver 3.01).

The thickness of basal layer to spinous layer and granular layer were measured at 10 sites of the epidermis under × 40 objectives. The final figure was the mean of all the measurements obtained for each biopsy specimen. Quantitation of AgNORs were performed using × 100 objectives. The number of AgNOR dots were counted in 100 nuclei in the basal keratinocytes from the randomly chosen region of the sample of the rats skin. The mean number of AgNORs per nucleus were calculated.

5. Data Analysis

For a comparison of the mean thickness of basal-spinous layer and granular layer, mean number of AgNORs per nucleus between the control and the electrical stimulation groups, a Student's t-test was used. The relationship between AgNOR number per nucleus of the basal keratinocytes and epidermal thickness was defined by calculating the Pearson product moment correlation coefficient. The statistical interpretation was based on a 0.05 significance test level. SPSS WIN (ver 10.0) software was used for the analysis.

RESULTS

1. Changes of the Epidermal Thickness

In the control skin, mean thickness of basal-spinous layer and thickness of granular layer were 12.72±1.64 μm and 8.59±0.73 μm, respectively. In the HVPC stimulated skin had mean thickness of the basal-spinous layer of 16.97± 1.25 μm and the granular layer of 7.47±1.29 μm. A Student's *t*-test showed a significantly higher mean thickness of the basal-spinous layer than control rats (t=5.046, $P<0.001$), whereas no significant difference was found mean thickness of the granular layer between the HVPC stimulation and the control group (t=1.902, $P>0.05$) (Fig. 1).

2. Changes of AgNORs

Clearly defined silver stained dots were visible in all specimens studied. In the control rats, a nucleus of the basal keratinocyte in epidermis had zero AgNOR in 24.17%, one AgNOR in 23.37%, two AgNORs in 25.88% and three AgNOR in 7.584%. In the HVPC stimulated rats, a nucleus of the basal keratinocyte in epidermis had zero AgNOR in 3.19%, one AgNOR in 28.15%, two AgNORs in 30.47%, three AgNOR in 11.73% and more than four in 3.43%. The mean AgNOR number per nucleus of the basal keratino-

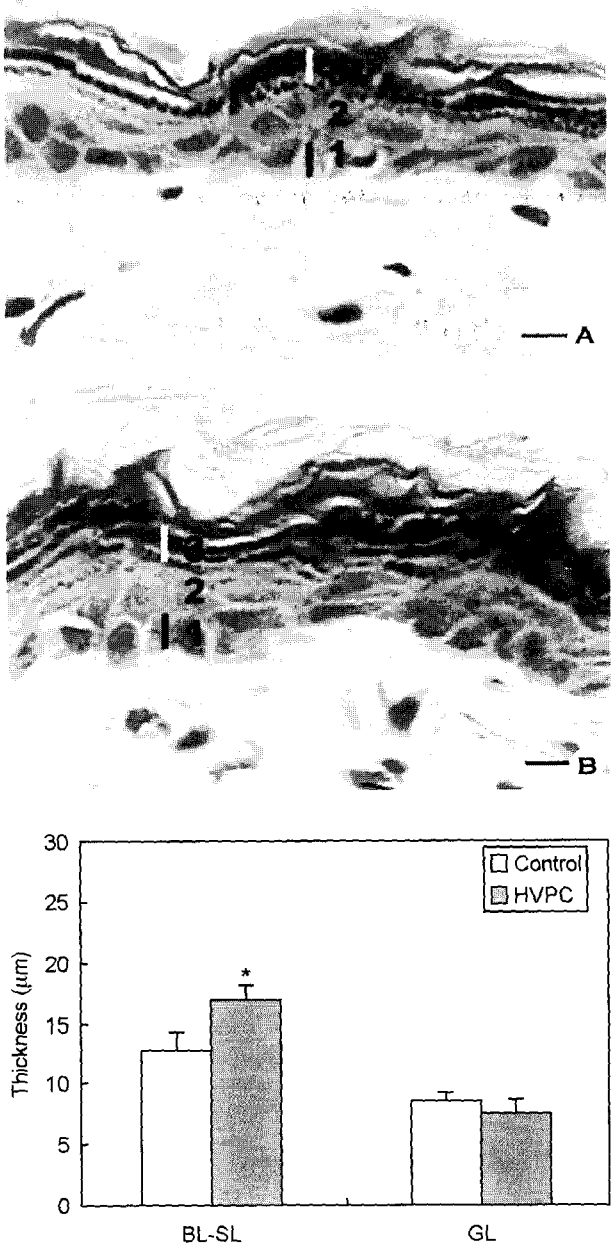

Fig. 1. Comparison of basal-spinous layer and granular layer in the control and HVPC stimulation groups. The panels are hematoxyline and eosin staining of skin from the control **(A)** and HVPC stimulated **(B)** rat's skin. The figure show an increase in the thickness of the basal-spinous layer (BL-SL) ($P<0.001$) of the epidermis can be observed in the HVPC stimulation group as compared with the control group, whereas the thickness of the granular layer (GL) is not affected. 1: basal layer, 2: spinous layer, 3: granular layer. Magnification: × 400; Scale bar: 10 μm.

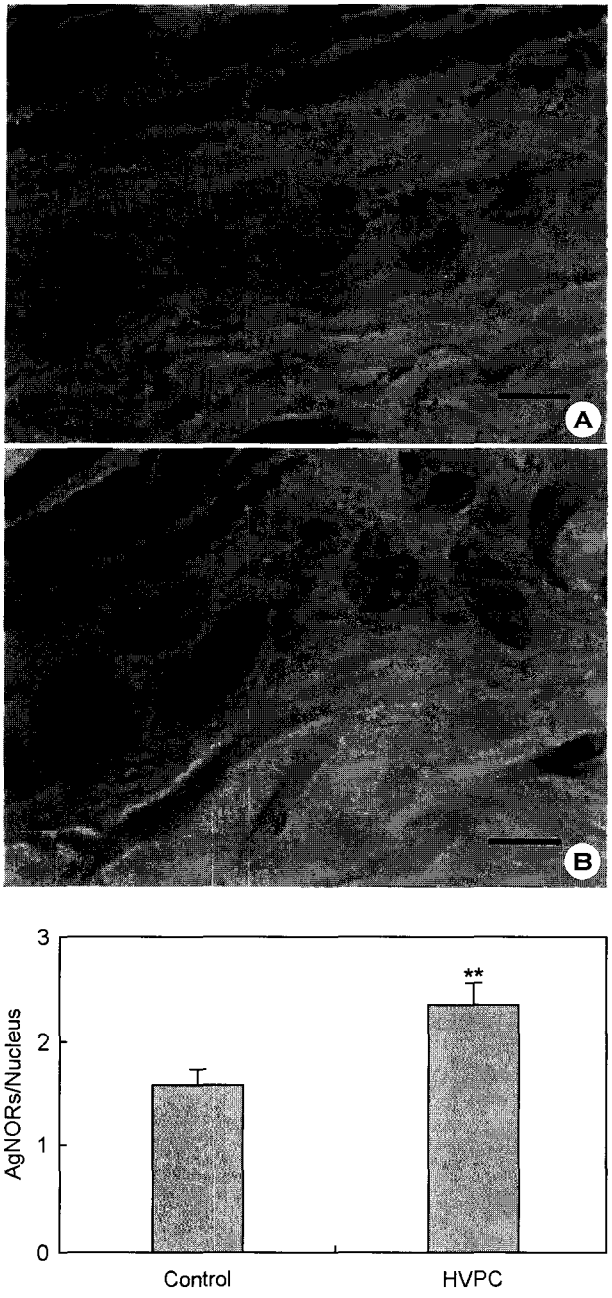

Fig. 2. Comparison of mean AgNORs number per nucleus in the basal keratinocytes in the control and the HVPC stimulated rat's skin. The panels show typical argyrophilic stained nucleolar organizer regions dots (arrow) in the nuclei of the basal keratinocytes from the control **(A)** and the HVPC stimulated **(B)** rat's skin. In the figure, an increase in the expression of nucleolar organizer regions in the basal keratinocytes can be observed in HVPC stimulation group as compared with the control group (P<0.001). Magnification: × 1000; Scale bar: 5 μm.

cytes in the control and HVPC stimulation groups were 1.58±0.15 and 2.36±0.20, respectively. Student's t-test showed a significantly higher mean AgNOR number in the

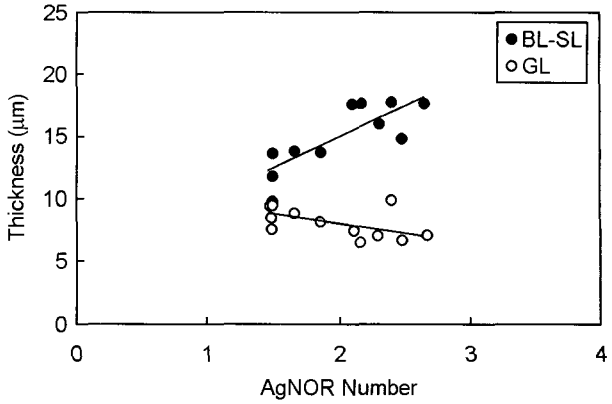

Fig. 3. Relationship between the mean AgNORs number per nucleus in the basal keratinocyte and the basal-spinous layer thickness and granular layer thickness in the aged rat skin. There were significantly positive correlation between the AgNOR number and the thickness of basal-spinous layer (r=0.80, P<0.01) and basal to granular layer (r=0.47, NS).

HVPC stimulated rats than control rats (t=7.527, P<0.001) (Fig. 2).

3. Correlation Between the Epidermal Thickness and AgNORs Number

There was a positive correlation between the mean AgNOR number per nucleus of the basal keratinocyte and thickness of the basal-spinous layer with a pearson product moment correlation coefficient of 0.80 (P<0.01). There was no correlation between mean AgNOR number per nucleus of the basal keratinocyte and thickness of the granular layer with pearson product moment correlation coefficient of 0.47 (p=NS) (Fig. 3).

DISCUSSION

This study was aimed at identifying proliferative activities of the keratinocytes that might explain the mechanisms of experimental and clinical effects of the HVPC on epidermis in aged skin. The HVPC stimulated rats showed a significantly higher mean thickness of the basal-spinous layer than sham control rats, whereas no significant difference was observed in the granular layer thickness. This finding suggests that the HVPC stimulation increased the thickness of spinous layer of epidermis in aged rat skin. The thickening of epidermis seems to affect the spinous cell layer whereas granular cell layer seem to be unaffected. Aged epidermis is decrease in the thickness by 10~50% between the age of 30 and 80, and resulting in atrophy to

affect the spinous cell layer (Sans et al., 1993; Lock-Andersen et al., 1997; Lavker, 1979). According to our data, HVPC resulted in a 32.48% increase the thickness of spinous layer compared to its corresponding sham control. These effects include acceleration of epithelialization and improvement of aged skin conditions.

The silver binds to non-histone nuclear proteins associated with the site of rRNA transcription. NOR can be histologically detected by silver stain as so-called AgNORs. Increases of NOR expression sites would be expected in actively proliferation of the cell. Therefore, it is clear that an increase in AgNOR number can seen in actively proliferating cells due to increased transcriptionally active rDNA sites, and in cells with a modal increase in chromosomes (Crocker et al., 1987). A correlation between AgNOR and cell proliferation indices have been reported (Giri et al, 1989). The estimation of AgNOR number is a marker of the keratinocyte proliferation activity. In these study, mean AgNOR expression number per nucleus of basal keratinocytes in the HVPC stimulated rats was significantly higher than control rats. In HVPC stimulated rats, many basal keratinocytes expressed more than one and two NORs in each nucleus. We also found that mean AgNOR number and thickness of the basal-spinous layer has a significant positive correlation. It has been reported that application of exogenous electrical signal can induce proliferation in human keratinocyte *in vitro* experiment (Hinsenkamp et al., 1997; Szabo et al., 2001; Manni et al., 2002; Manni et al., 2004). Our results indicate that the HVPC stimulated increased transcriptional activity in rDNA and rRNA production in nucleus, and stimulate protein synthesis in the basal keratinocytes. This finding strongly suggests that the HVPC stimulation accelerated proliferative activity of the basal keratinocyte in aged rats.

The molecular mechanism for the proliferation of keratinocytes by electrical signal is not defined precisely. The endogenous transdermal bioelectric potentials are the result of ionic pumps in the basal layer keratinocyte cell membrane (Barker et al., 1982). The current may play a role in the cellular activity (Illingworth et al., 1980; Foulds et al., 1983). We suppose that application of exogenous electrical signals may affect cellular metabolism by alteration of the electrical microenvironment of keratinocytes, and it may increase the proliferation of basal keratinocytes in skin.

Under our experimental conditions of HVPC increase of the spinous layer thickness by NORs expression in the basal keratinocyte in aged rats. These results suggest that the HVPC may increase the epidermal thickness with accelerate in proliferative activity of the basal keratinocytes in aged skin, It may help explain the mechanism of HVPC stimulation for improvement of aged skin. Further investigations with aged human skin and various parameters of electrical stimulation should be performed.

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