

Effects of Electrical Stimulation on the Nucleolar Organizer Region in Keratinocyte of Wounded Skin in Rat

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전기자극이 환취 창상 표피세포의 핵소체형성부위에 미치는 영향

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< Abstract >

The purpose of this study was to determine the effect of microamperage electrical stimulation on the number of argyrophilic nucleolar organizer region (AgNOR) in rat skin. Twenty four male Sprague-Dawley rats were divided into electrical stimulation and control group. Each animals hair on the back was removed. The electrical stimulation group received an positive rectangular pulsed electrical stimulation with 500 μ A, while the control group was given the same treatment without electricity. The rats were sacrificed at 4 and 7 day of stimulation, respectively. The biopsy specimens were fixed in formalin, embedded in paraffin and stained with silver nitrate. The AgNOR were counted using a light microscope and computerized image analysis system and calculated as the mean number of AgNOR per nucleus in the epidermal keratinocyte. In control skin, the mean AgNOR count of epidermal keratinocyte at 4 and 7 day were 1.67 and 1.72, whereas electrical stimulated rat had mean AgNOR counts of 2.0 and 2.14, respectively. A Student's t-test showed a significantly higher mean AgNOR number at 4 and 7 day in the electrical stimulated rats than control rats ($p < 0.05$). The microamperage electric current stimulation increased the epidermal AgNOR expression in incisional wound skin. These results suggest that the microamperage electrical stimulation may promote migration and proliferative activity of epidermal keratinocyte in surgical wound.

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<국문초록>

전기자극이 흰쥐 창상 표피세포의 핵소체형성부위에 미치는 영향을 규명할 목적으로 Sprague-Dawley계 수컷 흰쥐 24마리의 등에 15 mm의 절개창을 만들고 봉합한 후 각각 4일 7일 대조군 및 전기자극군에 각각 6마리씩 무작위로 배치하고 전기자극군 흰쥐의 등에 전극을 부착한 후 맥동빈도 0.5 pps, 전류량 500 μ A로 60분간 전기자극하였으며 대조군은 전극만 부착하고 전류를 통전시키지 않았다. 전자자극 후 등의 피부를 적출하여 포르말린에 고정한 후 파라핀 포매 절편을 제작하여 silver

colloid염색을 하였다. 이 표본을 400배율 현미경하에서 관찰하여 해당 AgNOR의 수를 계측한 후 해당 AgNOR의 분포와 해당 평균 AgNOR수를 산출하여 통계분석한 결과 창상유발 4일과 7일째의 전기자극군의 평균 AgNOR수가 대조군보다 통계학적으로 유의하게 높게 나타났다. 이러한 결과는 전기자극이 흰쥐 피부 창상연 표피세포에서 AgNOR 발현을 증가시켰음을 확인할 수 있었으며 이는 미세맥동전류자극이 창상연의 표피세포를 증식시키고 있음을 시사하고 있다.

I. INTRODUCTION

Wound healing is a complex series of biologic events including coagulation, inflammation, fibroplasia, matrix deposition, epithelialization, contraction and angiogenesis (Hunt, 1990). Healing of skin wound progresses through three serial phases as an inflammatory phase, a proliferative and reparative phase and a remodeling phase (Pollack, 1984). There is overlap of these phases of healing, and the entire process can last several months. The inflammatory phase begins immediately after injury and lasts 2 to 5 days. The proliferative phase begins 2 days after injury and lasts 3 weeks. The epithelialization commences immediately after injury as a priority for the body to protect itself from invasion by organisms and occurs concurrently with the inflammatory phase. The epidermal keratinocytes respond to signals from the macrophages, neutrophils, and current of injury. The responding epidermal keratinocytes proliferate and migrate from the wound edges and dermal appendages until to resurface the open wound. The ability of a wound to epithelialize is important to healing process. The newly formed epithelial layer protects the wound from infection (Bang et al, 1998). If the proliferative and migratory ability of the epidermal keratinocytes are impaired, wound have important clinical consequences such as delayed healing

or non healing (Seiler and Stahelin, 1994).

Recently, the Agency for Health Care Policy and Research (AHCPR) recommended electrical stimulation therapy for patients with stage III or IV pressure ulcers that are not healing to more conventional treatments (Bergstrom et al, 1994). Electrical stimulation for biological growth and repair are well known (Brown et al, 1988; Cruz et al, 1989). Although few reports exist concerning the effect of electrical stimulation on epithelialization of skin wound. Alvarez et al (1983) reported that the direct current electrical stimulation increased collagen synthetic capacity and accelerated the rate of wound epithelialization. Agren et al (1994) reported that a pulsed electrical stimulation accelerated epithelialization of burn wound.

Silver-stained nucleolar organizer regions (AgNORs) have been widely used as a marker of cellular activity and proliferation (Ghazizadeh et al, 1997). The estimation of epidermal AgNORs number are markers of epidermal proliferation activity (Heinisch and Wozel, 1995). We therefore examined the epidermal AgNOR expression after electrical stimulation using the rat skin wound. The effect of electrical stimulation on the activity of epidermal keratinocytes in wound skin have not been demonstrated. Consequently, the purpose of this study was to ascertain whether microamperage electrical stimulation affects in the proliferative activity of epidermal keratinocytes in surgical wounded skin.

II. MATERIALS AND METHODS

1. Animals

Twenty-four male Sprague-Dawley rats weighing 180-220 g were used. The rats were maintained under clean conventional conditions, under which they were fed standard rat chow (Samyang Feedstuff, Samyang Feedstuffs Co., Ltd., 400-3 Woosan-dong, Wonju, Kangweon, Corea). They had access to tap water ad libitum, and were kept under a 12 h light/dark cycle at a constant temperature of $24 \pm 2^\circ\text{C}$.

2. Wound Preparation

Before making an incision, we shaved the skin on the dorsal aspect of the lumbar region, washed it with alcohol, dried it, and anesthetized rat with 40 mg/kg of sodium penthotal intraperitoneal injection. We then placed marks 15 mm apart on the skin, 20 mm to the left of vertebral column. We cut a 15 mm length of full-thickness incision with a sterile scalpel #11. The incisional wound was sutured with a sterile 3-0 silk (Ethicon Inc., Somerville, New Jersey, USA). The wound area was sponges clean with a sterile gauze pad, and the rat was observed until it had recovered fully from the anesthesia. The wounds were not covered or bandaged.

3. Electrical Stimulation

The rats were placed in a clear plastic restrainer and rested comfortably. The wound area of rat skin were covered with saline-soaked gauze pads (2×2 cm), and the electrodes pads were placed on the gauze and secured in a restrainer. The salined saturated dispersive pad electrode were placed on the rat's back and secures with pads. Electric line cords were inserted through the window in the restrainer. The electrically stimulated rats received electrical stimulation with a current intensity of $500 \mu\text{A}$ at 0.5 pps through the electrodes for a duration of 60 minutes using a commercial microcurrent stimulator

(Micro PlusTM, BioMedical Life Systems, Inc., 1120 Sycamore Avenue, Suite F, Vista, California 92083, USA). The shape of the applied waveform was biphasic rectangular. Control rats received a sham treatment.

4. Tissue Sampling and AgNOR Staining

The rats were induced anesthesia with ethyl ether. 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. Following tissue sampling, the rats were sacrificed by ether anesthesia. The fixed tissues were dehydrated by ascending graded alcohol series, cleared with xylene using an automatic tissue processor (Citadel 1000, Shandon, Life Sciences International Ltd., Astmoor, Rumcorn, England, WA7 1PR). The tissue sample was embedded in paraffin and cut into $4 \mu\text{m}$ thick serial sections using a rotary microtome (Rotary Microtome HM 340E, Microm Laborger te GmbH, Robert-Bosch-Strasse 49, D-6909 Walldorf, Germany). For each rat tissue sample, serial sections were stained with colloidal silver.

5. Quantification of AgNORs

Quantification of AgNORs were performed using a computerized image analysis. For video image analysis, a light microscope (Olympus BX 50, Olympus Optical Co., Ltd., 2-43-2, Hatagaya, Shibuya-Ku, Tokyo, Japan) was linked to a CCD camera (IK-642K Toshiba CCD color camera, Toshiba Co., 1-1-1 Shibatori, Minato-Ku, Tokyo, Japan), and an image processing and analysis system (Image-Pro[®] Plus, Media Cybernetics, Inc., 8484 Georgia Avenue, Silver Spring, MD 20910, U.S.A.). The software used in this system was a WIN98, along with a computerized image analysis software Image-Pro[®] Plus (ver 3.01). Quantification was performed using $\times 40$ objectives. The number of AgNOR dots were counted in 100 serial fields from the randomly chosen region of the sample of the rats skin. The mean number of AgNORs per nucleus were calculated.

Table 1. Mean and standard deviation of AgNOR number per nucleus in epidermis in the electrical stimulated and control rats (Mean \pm SD).

Group	n	7 days	4 days
Control	6	1.72 \pm 0.34	1.67 \pm 0.31
Electrical stimulation	6	2.14 \pm 0.19	2.00 \pm 0.32

Table 2. Group t-test of mean AgNOR number per nucleus in epidermis in the electrical stimulated and control rats.

	df	DM	SE	t	p
4 days	10	.33167	.13487	2.459	.034
7 days	10	.41690	.15849	2.630	.025

Table 3. Distribution (%) of AgNOR number per nucleus in epidermis in the electrical stimulated and control rats at 4 and 7 days of wound.

Group	No. of AgNOR per Nucleus							
	0	1	2	3	4	5	6	7
Control 4 days	25.79	17.29	22.55	18.04	8.64	3.57	3.00	1.12
Control 7 days	17.70	27.51	29.51	14.90	5.30	2.99	1.55	0.54
ES 4 days	15.38	18.21	26.70	28.30	6.78	3.21	1.42	0.00
ES 7 days	15.05	18.03	29.52	22.93	9.46	4.33	0.49	0.19

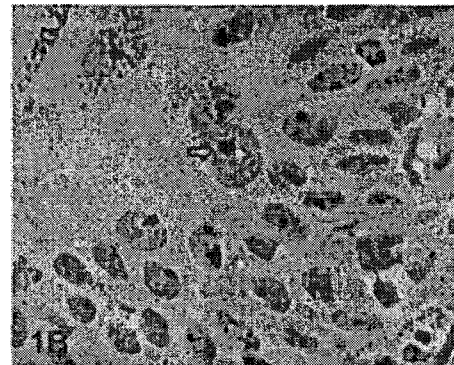
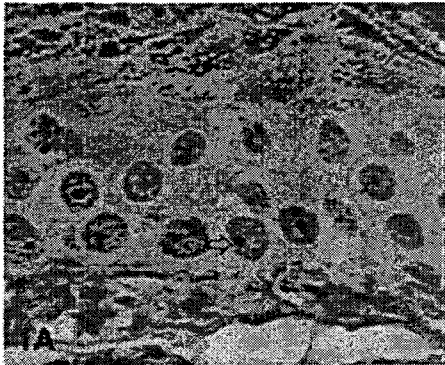


Fig 1. The dark brown stained AgNOR dots (arrow) in epidermis of the control (A) and the electrically stimulated rat skin (B) at 4 days of incision. The electrically stimulated skin showed more numerous AgNOR expression in nucleus of epidermal keratinocytes than control skin. Silver colloid stained, x400.

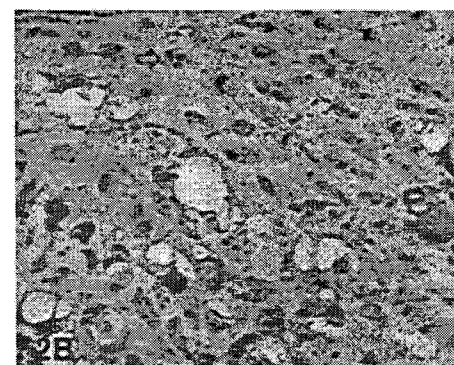
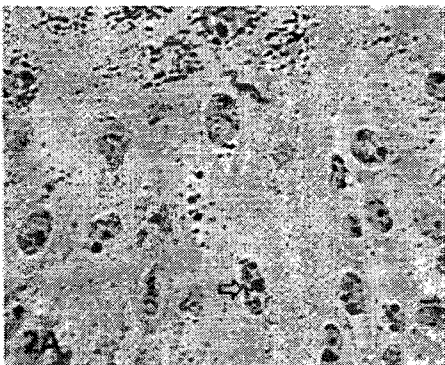


Fig 2. The dark brown stained AgNOR dots (arrow) in epidermis of the control (A) and the electrically stimulated rat skin (B) at 7 days of incision. The electrically stimulated skin showed more numerous AgNOR expression in nucleus of epidermal keratinocytes than control skin. Silver colloid stained, x400.

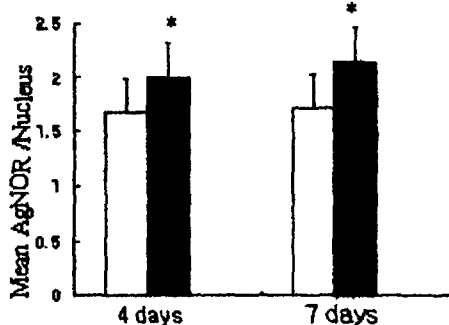


Fig 3. Comparison of mean number of AgNORs in nucleus of epidermal keratinocyte on 4 and 7 days of incisional wounded in control (open bar) and electrical stimulated (closed bar) rats. Bars indicate standard deviation. A t-test showed a significantly higher mean AgNOR number in the electrical stimulated rats than control rats at 4 and 7 day post wounded ($p < 0.05$).

6. Data Analysis

For a comparison of the mean number of AgNORs per nucleus between the electrical stimulation and the control groups, a Student's t-test was used. The statistical interpretation was based on a 0.05 significance test level. SPSS WIN (ver 7.5) software was used for the analysis.

III. RESULTS

Clearly defined silver stained dots were visible in all specimens studied (Fig. 1, 2). In control skin, the mean AgNOR count of epidermal keratinocyte at 4 and 7 day were 1.67 and 1.72 respectively. In electrical stimulated rat had mean AgNOR counts of 2.0 and 2.14 respectively (Table 1). Student's t-test showed a significantly higher mean AgNOR number at 4 and 7 day in the electrical stimulated rats than control rats ($p < 0.05$) (Table 2) (Fig. 3).

In control rats, a nucleus of keratinocyte in epidermis had zero AgNOR in 25.79%, 17.70%, one AgNOR in 17.29%, 27.51%, two AgNORs in 22.55%, 29.51% and three AgNOR in 18.04%, 14.90% at 4 and 7 days of wound, respectively. In electrical stimulated rats, a nucleus of keratinocyte in epidermis had zero AgNOR in

15.38%, 15.05%, one AgNOR in 18.21%, 18.03%, two AgNORs in 26.70%, 29.52% and three AgNOR in 28.30%, 22.93% at 4 and 7 days of wound, respectively.

IV. DISCUSSION

Nucleolar organizer regions (NORs) are chromosomal segments in which ribosomal RNA is encoded, and they are thus responsible for the development of the RNA-containing nucleolus into which the NORs project on large loop of DNA. NORs are located on each of the short arms of the acrocentric chromosomes 13, 14, 15, 21 and 22, and therefore 20 may be seen in a normal diploid whole cell (Fakan and Hernandez-Verdun, 1986).

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 count can be seen in actively proliferating cells due to increased transcriptionally active rRNA sites, and in cells with a modal increase in chromosomes (Crocker and Skilbeck, 1987). A correlation between AgNOR counts and cell proliferation indices have been reported (Giri et al, 1989). The estimation of AgNOR number in epidermis is a marker of keratinocyte proliferation activity.

We therefore examined the epidermal AgNOR count after microampere electrical stimulation using the rat cutaneous wound. In this experiment, the mean number of AgNORs in the electrical stimulated rats was significantly increased than the control rats. This result showed that the electrical stimulation increases the expression of NOR in epidermal keratinocytes. It suggests that the electrical stimulation promotes the proliferative activity of basal keratinocytes in epidermis.

In surgical wounds that are sutured, epidermal keratinocyte proliferation and migration begins within the first 24 hours. Goodman et al (1983) reported that weak electromagnetic fields increased the mRNA synthesis in dipteran salivary gland cells. The epidermal keratinocyte migration influenced by electrical

stimulation. Luther et al (1983) described a galvanotropic response of epithelial cells. They found the direction of growth and orientation of cell can be influenced by externally applied direct current electric fields. Jaffe and Venable (1984) have shown that the cellular movement may be enhanced by voltage gradient. Cooper and Schliwa (1985) reported that fish epidermal keratinocytes migrated under direct current electric fields of 0.5-15 V/cm. Bereiter-Hahn and Luers (1998) reported that the keratinocytes change their direction of movement by exposure to DC electric fields. Hinsenkamp et al (1997) reported that the low frequency pulsed electrical current enhanced migration and proliferation of keratinocyte. Nishimura et al (1996) found that epidermal keratinocytes migrate towards the direct current electric fields, exhibiting galvanotaxis.

The molecular mechanism for the proliferation and migration of keratinocytes by electrical stimulation is not known precisely. But it is well known that the wounded skin has a current of injury which generated by the skin epithelium. 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 wound healing process (Illingworth and Barker, 1980; Foulds and Barker, 1983). We suppose that the external application of electric potentials which similar to the current of injury may accelerate the cellular metabolism by alteration of the electrical microenvironment of keratinocytes, and it may increase the proliferation and migration of epidermal keratinocytes in wounded skin.

Further investigations with many groups of animals and various parameters of electrical stimulation should be performed to substantiate the optimal parameters of electrical stimulation for affect to epidermal keratinocytes.

V. CONCLUSION

The purpose of this study was to determine the effect of microamperage electrical stimulation on the number of AgNOR in rat skin. The results of this study revealed a statistically significant higher mean number of AgNOR

in electrical stimulated rats compared to that in control rats at 4 and 7 days of wounded skin.

These results suggest that the microamperage electrical stimulation may promote migration and proliferation activity of epidermal keratinocyte in surgical wound.

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