원 저

Immunohistochemical Study on the Inflammation-related Proteins in the Ankle Joint of Complete Freund's Adjuvant-injected Rat by Electroacupuncture Stimulation

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국문초록

전침에 의한 Complete Freund's Adjuvant유발 관절염모델의 거퇴관절 내 염증관련 단백질에 대한 면역조직화학적 연구

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목적 : 만성 염증성 질환에 대한 전침효과를 알아보기 위해 complete Freund's adjuvant (CFA) 유발 관절염 모델에서 염증관련 단백질의 변화를 살펴보았다.

방법 : Sprague-Dawley계 흰쥐의 족부에 CFA를 주사한 다음 3일 간격으로 2 Hz, 15 Hz 및 120 Hz 전침자극을 주며 부종 형성여부를 plethysmometer로 측정하여 판정하였으며 30일 째 거퇴관절을 취하여 4% paraformaldehyde에 고정하고 EDTA용액에서 탈회시켜 파라핀연속 절편을 얻어 NF-κB를 비롯한 5종의 염증관련 단백질의 발현을 면역조직화학적으로 살펴보았다.

결과 : 관절연골내 면역반응 중 연골기질은 반응이 없거나 약하고 연골세포는 NF-κBp65, I-κBα, iNOS반응이 강하며 특히 유리연골층에서 더 현저하였으나 염증 및 전침자극에 따른 변화는 없었다. 관절낭에서 면역반응을 살펴보면 염증유발시 활액세포의 면역반응세포는 I-κBα가 감소한 반면 iNOS, IL-1β는 증가하며 특히 iNOS 증가가 현저하였으며 전침자극에 의해 iNOS가 감소하였다. 활액막조직에서 모든 면역반응이 증

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가하며 특히 NF-κBp65, I-κBα, iNOS 반응이 현저한데 전침자극에 의해 IL-1β를 제외한 모든 반응이 감소하였다.

결론 : 만성 염증성 동물모델의 거퇴관절 내 염증관련 단백질은 관절연골보다 관절낭에서 큰 변화를 보이며 전침처치에 의해 이들 단백질 발현이 억제되는 것으로 보아 전침이 만성 염증성 질환에 효과적임을 알 수 있다.

Key words : Electroacupuncture, Arthritis, NF-κB, I-κB, iNOS, TNF-α

I. Introduction

The nuclear factor (NF)-κB, a pivotal transcription factor in inflammatory response, activation plays an important role in expression of inflammatory factors such as inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF)-α and interleukin (IL)-1[1]. The NF-κB is normally inactive, bound by members of the inhibitory (I)-κB family, including I-κBα, in the cytoplasm. NF-κB is dissociated from I-κB and translocate into the nucleus where it induces transcriptional up-regulation of various proinflammatory mediators that contribute to organ injury[2]. Thus the blocking of NF-κB activation may be an effective strategy in the treatment of inflammation-induced organ injury.

Adjuvant arthritis develops inflammation in the articular tissue such as an inflammatory synovitis and ultimately leads to erosive joint and bone destruction[3]. The complete Freund's adjuvant (CFA)-induced arthritis in rat been used for many years for evaluation of anti-rheumatoid arthritic treatments as a reliable arthritic animal model[4]. With biochemical studies, the analysis of the morphological and immunohistochemical changes also constitutes an important aspect of arthritis characterization. Current treatment for arthritis is not largely satisfied with glucocorticoids and non-steroidal anti-inflammatory agents because of numerous systemic side effects from long-term use[5].

Electroacupuncture (EA) has been clinically used as a therapeutic means for the mitigation of acute or chronic inflammatory diseases in oriental medicine. Numerous studies show that EA stimulation has analgesic effects which induce selectively the release of enkephalins and dynorphins in various experimental pain models[6-7]. But EA may affect chronic systemic disease such as arthritis by means of inhibitory activity towards the inflammatory process in the joints of animal model. Therefore, we investigated the effects of EA stimulation for treating rheumatoid arthritis and suppressing the development of CFA-induced arthritis using immunohistochemical methods.

II. Materials and Methods

1. Animals

Male Sprague-Dawley rats, weighing approx. 120 g, were obtained from Hyochang Science, Daegu, Korea. Rats were housed under constant environmental conditions at 22℃ and a 12-hour dark-light cycle, and were fed a commercially obtained diet and allowed tap water *ad libitum* starting 2 weeks before and throughout the study. The experimental procedures were conducted under the ethical guidelines for investigations of experimental pain in conscious animals[8].

2. CFA Injection and EA Stimulation

Rats were injected subcutaneously with 150 ㎕ of CFA (1 ㎎ *Mycobacterium tuberculosis* per 1 ㎖ Sigma, St. Louis, MO, USA) into the plantar surface of the hindpaw. For EA stimulation at 2 Hz, 15 Hz and 120 Hz, rats were partially restrained in a plastic holder. Two stainless-steel needles with 0.25 ㎜ diameter were inserted in each hindleg at those acupoints corresponding to Zusanli (ST36, 5 ㎜ lateral to the anterior tubercle of the tibia) and Sanyinjiao (SP6, 3 ㎜ proximal to the medial malleolus) in humans and were connected to an electric stimulator (SM-60 Saechang, Seoul, Korea). The intensity was set at 1 ㎃ and was increased stepwise to 2 ㎃ and 3 ㎃ and each step lasted 10 min. EA stimulation was repeated with 3-day intervals for a total of 30 days after CFA-injection. Normal groups were injected with 150 ㎕ of phosphate buffered saline (PBS, pH 7.4) only, and were not treated with EA.

3. Measurement of Body Weight, Paw Swelling and Tissue Preparation

Body weight was determined at the various time points and paw edema also measured by a water-displacement plethysmometer (Ugo-Basile, Comerio, Italy). Thirty days after CFA-injection, the ankle joint of paw was obtained from the normal, CFA-injected control and EA-treated experimental rats and fixed in 4% paraformaldehyde in PBS for 24 hours. Tissues were decalcified with 4% EDTA solution and dehydrated in a graded ethanol series and embedded in paraffin. Serial 5 ㎛ thick sections were prepared.

4. Immunohistochemistry

After deparaffinized in 58℃ xylene, the sections were exposed for 30 minutes to 0.3% methanolic hydrogen peroxide, followed by washing with PBS. Tissues were then treated with goat normal serum at room temperature for 30 minutes followed by treatment with anti-NF-κBp65, I-κBα, TNF-α, iNOS and IL-1β (Santa Cruz, CA) diluted for 1:500 in moisture chamber for 16 hours at 4℃. After washed by PBS, tissues were incubated with the secondary antisera, biotinylated anti-rabbit Ig G for 30 minutes, followed by washing with PBS. These sections were further incubated in avidin-biotin-peroxidase complex kit (Vector, Burlingame, CA, USA) for 60 minutes at room temperature. Diaminobenzidine substrate kit (Vector) for peroxidase was applied. For the controls, treatment with primary and secondary antibodies was omitted.

5. Data analysis

Data were expressed as mean±SEM. Calculations of means, standard errors and Student's *t*-test were made using SigmaPlot version 6.0 software (SPSS, Chicago, IL, USA). $P<0.05$ was considered statically significant.

Ⅲ. Results

1. The Body Weight and Paw Swelling

The significant difference showed between normal and CFA-injected rats with or without EA stimulation. But there was no difference between control and EA-treated rats except 3 and 12 day after CFA-injection as shown in Fig. 1. Paw swelling of ipsilateral hindpaw of CFA-injected rats was larger than the size of the pre-injection baseline value throughout the 30 days after CFA-injection. However, as illustrated in Fig. 2, EA stimulation at 2 Hz, 15 Hz and 120 Hz produced significant anti-edema effects compared to the CFA-injected control rats.

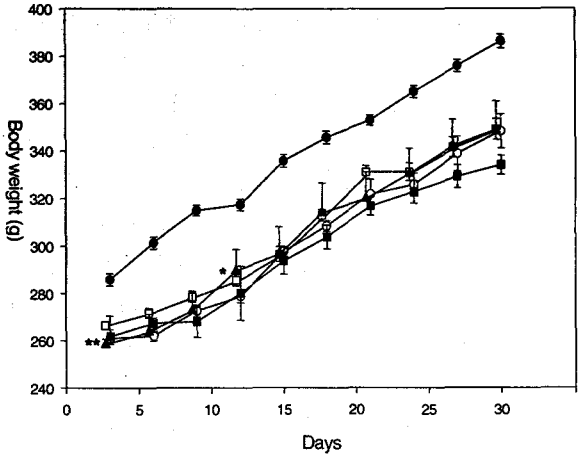

Fig. 1. The whole body weight in the normal (●), CFA-injected control (○) and EA-treated rats (▲, 2 Hz; □, 15 Hz; ■, 120 Hz). Each point indicates the means±SEM (n=8)
* P<0.05 and ** P<0.005 indicate significant differences from control group.

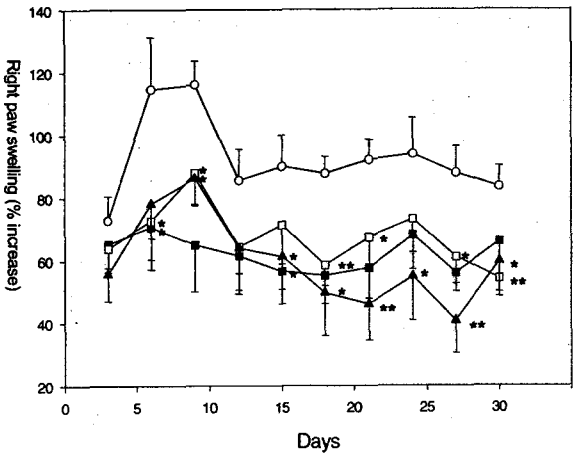

Fig. 2. Anti-edematous effects of EA at different frequencies (○, control; ▲, 2 Hz □, 15 Hz ■, 120 Hz) in CFA-injected rats. Each point indicates the means±SEM (n=8). EA treatment induced a significant inhibition of CFA-induced edema compared with controls
* P<0.05 and ** P<0.005 indicate significant differences from control group (Cited from reference 9).

2. Immunohistochemical Analysis

To investigate the expression of inflammation-related protein in the ankle joints after induction of inflammation with or without EA stimulation, we determined NF-κBp65, I-κBα, TNF-α, iNOS and IL-1β immunoreaction. The results of the immunohistochemical study on the inflammation-related protein are outlined in Table 1 and Figs. 3-4. The immunohistochemical analysis showed that while weak or absent immunoreaction showed in the matrix of articular cartilage, more intensive for NF-κBp65, I-κBα, iNOS were observed in chondrocytes of articular cartilage, especially in the zone of hyaline cartilage. But there were no significant difference between CFA-injected rats and EA-treated rats.

While I-κBα immunoreaction in the synovial lining cells was decreased in rats challenged with CFA, the immunoreaction for iNOS and IL-1β tended to increase compared to normal rats. But the iNOS expression significantly decreased in the EA-treated rats compared to CFA-injected rats. All immunoreaction for inflammation-related protein examined in the subsynovial tissue tended to increase in CFA-injected rats compared to normal rats. Much more intensive expression of NF-κBp65, I-κBα and iNOS were detected in subsynovial tissue of CFA-injected rats. However, a significant decline of these immunoreaction was detectable in the EA-treated rats.

Table 1. Inflammation-related protein expression in the ankle joints of the rats with CFA-induced arthritis by immunohistochemistry

Stains	Region	Normal rat	Control rat	EA-treated rat		
				2 Hz	15 Hz	120 Hz
NF-κBp65	MAC	0	0	0	0	0
	CHC	0,++	0,++	0,++	0,++	0,++
	CCC	0	0	0	0	0
	SLC	0	0	0	0	0
	SST	0	0-+++	0	0	0
I-κBα	MAC	0-+	0-+	0-+	0-+	0-+
	CHC	++++	++++	++++	++++	++++
	CCC	+++	+++	+++	+++	+++
	SLC	0-+++	0-++	0-++	0-++	0-++
	SST	0	0-+++	0	0	0
TNF-α	MAC	0	0	0	0	0
	CHC	0	0	0	0	0
	CCC	0	0	0	0	0
	SLC	0-+	0-+	0-+	0-+	0-+
	SST	0	0-++	0	0	0
iNOS	MAC	0	0	0	0	0
	CHC	+++	++++	+++	+++	++++
	CCC	+-++	+-++	+-++	+-++	++
	SLC	+	+++	+-++	+-++	++
	SST	0	0-+++	0-++	0-++	0-++
IL-1β	MAC	0-+	0-+	0-+	0-+	0-+
	CHC	0	0	0	0	0
	CCC	0	0	0	0	0
	SLC	0	0-+	0-+	0-+	0-+
	SST	0	0-+	0-+	0-+	0-+

0-++++ indicate the relative intensity of the reaction: ++++, very intense; +++, intense; ++, moderate; +, weak; 0, absent. MAC, matrix of articular cartilage; CHC, chondrocyte in the zone of hyaline cartilage CCC, chondrocyte in the zone of calcified cartilage; SLC, synovial lining cell; SST, subsynovial tissue.

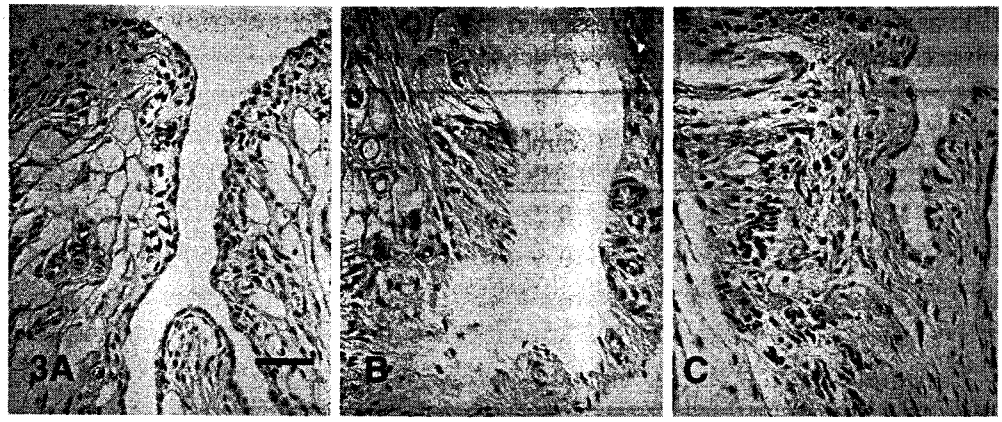

Fig. 3. Photomicrographs showing the articular capsule of the ankle joint in the normal (A), CFA-injected control (B) and EA-treated experimental rats (C) on day 30 post arthritis onset. Note marked NF-κBp65 immunoreaction in the subsynovial tissue of CFA-injected rats compared to other rats. Scale bar = 50 ㎛

Fig. 4. iNOS immunoreaction in the normal (A), CFA-injected control (B) and EA-treated experimental rats (C) on day 30 post arthritis onset. Note marked iNOS immunoreactionin in the synovial lining cells of CFA-injected rats compared to other rats. Scale bar = 50 ㎛

Ⅳ. Discussion

The transcription factor NF-κB serve as critical regulators of the inducible expression of many inflammatory genes[10]. I-κB degradation is closely related to NF-κB activation. The proinflammatory cytokines initiate a signaling cascade leading to the activation of I-κB kinase, which phosphorylates I-κB at specific N-terminal serine residues[11]. The phosphorylated I-κB is selectively degraded by the 26S proteasome. The activation of NF-κB is associated with the production of inflammatory enzymes such as iNOS and COX-2[12].

NF-κB plays also a pivotal role in pathogenesis in general arthritis. The NF-κB immunoreaction is increased in synovial intimal lining of arthritic joint[13]. NF-κB immunoreaction is found mainly in the cytoplasm, not the nucleus, of the synovial lining cells of induced-synovitis[14]. Intra-articular injection of lipid A induced NF-κB expression in cartilage and synovium and quantitative analysis in cartilage may be useful to evaluate cartilage degeneration[15]. Blocking NF-κB is a potential strategy for preventing chronic inflammatory disease and selective inhibition of its activation offers an effective therapeutic approach[16].

As for the immunohistochemical studies in the present, intensive I-κBα immunoreaction were observed on the chondrocytes of articular cartilage. But different immunoreaction of NF-κBp65 and I-κBα between normal and CFA-injected rats were merely observed in the subsynovial tissue and decline of these reaction were detected in the EA-treated groups. There were no exact evidence for translocation of NF-κBp65 into the nucleus by immunohistochemical analysis. However, these results obtained from immunohistochemistry showed a different expression of NF-κB and I-κB in the subsynovial tissue by EA stimulation.

NF-κB-induced gene expression contributes significantly to the pathogenesis of inflammatory diseases such as arthritis[17]. Inhibition of NF-κB activation with an inhibitor of I-κB degradation eliminate iNOS expression and TNF-α synthesis. NO is relevant to the pathogenesis of joint swelling in the inflammatory arthritis[18]. Several immunohistochemical studies reported that iNOS is most strongly expressed in the synovial lining layer, subsynovium, vascular smooth muscle and chondrocytes and in additional expression in the endothelial cells and synovial fibroblast with rheumatoid arthritis[19-20].

Inflammatory subsynovium and chondrocytes are major source of increased NO production and iNOS expression in patient with inflammatory arthritis[20]. In the present study, iNOS expression was detected mainly in the chondrocytes of the articular cartilage. But significant increase of this reaction in CFA-injected rats were observed in the synovial lining cell and subsynovial tissue and tended to decreased in the EA-treated rats.

TNF-α is a principal regulator of inflammation and mediates induction of other cytokines, COX-2, prostaglandins and metalloproteinases, which leads to cartilage degradation in arthritis. TNF-α, COX-2, and prostaglandin E_2 play a critical role in the pathophysiology of arthritis. TNF-α is mainly expressed in the region with a marked infiltration of inflammatory cells in the arthritic joint[21].

Other cytokines such as IL-1β and IL-6 play distinct roles in the pathological mechanisms of synovial tissue proliferation and joint destruction in arthritic joint. The immunoreaction for TNF-α and IL-1β were mainly observed in the articular capsule, immunoreaction for TNF-α in the synovial lining cells and IL-1β in articular capsule slightly increased in CFA-injected rats compared to normal rats. The different expression for TNF-α between CFA-injected rats and EA-treated rats showed only in the synovial lining cells of articular capsule.

The body weight, paw volume of the joint are exploited as an assessment method addressing arthritic symptoms. Although there was no difference in the whole body weight between CFA-injected rats and EA-treated rats, different immunoreactions were detected in the EA-treated rats compared to CFA-injected rats with significant decline of paw swelling. The results obtained from immunohistochemistry showed a different induction of inflammation-related protein in the ankle joint, especially in articular capsules. These observations are consistent with a critical role of NF-κB activation and other enzyme and cytokines participating process of inflammation. In conclusion, the results reported here suggest that EA stimulation inhibits expression of inflammatory regulators. Thus EA treatment may be useful in the therapeutic menas for chronic inflammatory disease.

V. References

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