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Paw Edema was Reduced in Carrageenan Induced Acute Inflammation in Stat4 Deficient Mice

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Abstract

Signal transducer and activator of transcription 4 (STAT4) is one of the important mediators in generating inflammation and immune responses. To address the role of Stat4 in carrageenan induced acute inflammation, we performed paw edema measurement and 7.4 k mouse cDNA microarray analysis in carrageenan induced acute inflammation in Stat4 knockout (-/-) mice. Male BALB/c (n=8) and Stat4 -/- (n=5) were used and paw edema was induced with injection of 30 μL of 1% carrageenan into plantar surface of right hind paw. Next, we isolated the mRNA in mouse whole brain and analyzed cDNA microarray profiles for the changes of the brain expression in Stat4 -/- mice. Interestingly, the increase in paw volume of Stat4 -/- mice was reduced by about 30% as compared to that of wild type. The cDNA microarray analysis revealed the altered expressions of several cytokines (*Tnf*, *Il6*, and *Il4*) and pain-associated proteins (*Ptgs2*, *Gabra6*, and *Gabbr1*) in Stat4 -/- mice. Our results suggest that Stat4 may be related to the inhibitory responses on carrageenan induced acute inflammation.

Keywords: Stat4 knockout mice, Carrageenan, Inflammation, Paw edema, Brain, Microarray

Signal transducer and activator of transcription 4 (STAT4) is one of the key molecules during the immune and inflammatory responses. STAT4 is phosphorylated in response to interleukin 12 (IL12) and related IL12 signaling pathway[1]. Kaplan *et al.*[2] reported that Il12 induced increases in the production of interferon gamma (Ifng) cellular proliferation and natural killer (NK) cell cytotoxicity are abrogated in lymphocytes from Stat4 -/- mice. Il12 is a pro-inflammatory cytokine, and recently, patients receiving IL12 as an immune therapy for several cancers appeared hyperalgesia[3,15-17].

Carrageenan is a high molecular weight polysaccharide and has been used to generate acute inflammation and hyperalgesia animal model[4]. Acute inflammation of carrageenan may be induced by the activation of natural killer cells, phagocytes, and other lymphocytes[5]. However, there has been no information about the effect of Stat4 in carrageenan induced acute inflammation.

It is suggested that the periphery and central immune system plays an important role in hyperalgesia and analgesia[6]. In the early stage of inflammation, several endogenous mediators are produced including pro-inflammatory cytokines {IL1, IL6, and tumor necrosis factor (TNF)}, nerve growth factor, and prostaglandins[7-9]. In parallel, anti-inflammatory cytokines such as IL4, IL10, IL13, and IL1 receptor antagonist are also produced, and reduced the hyperalgesic effects of pro-inflammatory cytokines initially produced[10-13]. Therefore, inflammatory pain may, in part, be regulated by interaction between hyperalgesic and analgesic mediators.

In this study, to investigate the role of Stat4 in acute inflammation, we performed paw edema measurement and 7.4 k mouse cDNA microarray analysis in Stat4 knockout (-/-) mice using carrageenan induced acute inflammation model.

Carrageenan Induced Paw Edema

It was well-known that subcutaneous injection of carrageenan was induced acute inflammation and edema[14]. In the present study, subcutaneous injection of carrageenan led to increase in paw volume that was maximal after 1 h in wild type mice (Fig. 1). This result supports previous reported experiments. However, carrageenan-induced paw edema exchange ratio was significantly reduced in Stat4 -/- mice, compared to wild type mice at 1, 2 and 3 h (Fig. 1, $P<0.05$).

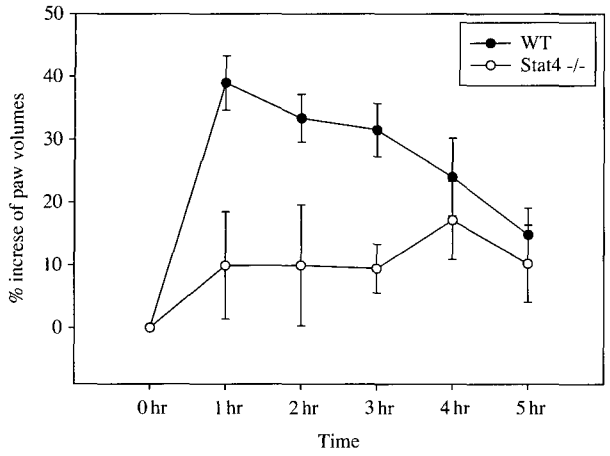

Fig. 1. Carrageenan induced mouse paw edema. Paw volume was measured at 0, 1, 2, 3, 4, and 5 hr after subcutaneous injection of carrageenan or saline. The results are expressed by the mean ± SEM of five animals per group. #, statistically significant difference compared with the WT group ($P < 0.05$). WT, wild type mice; Stat4 -/-, Stat4 knockout mice.

Gene Expression Analysis

To examine the relation between the brain gene expression and potential functional consequence, we performed the cluster analysis using the Tree View program (http://genopole.toulouse.inra.fr; http://rana.lbl.gov/EisenSoftware.htm). Forty five genes were selected for cluster analysis, and 4 clusters were made. Gene names and accession numbers are listed in Table 1. Cluster A showed the upregulated genes in wild-type mice, but not in Stat4 -/- mice (wild type-specific carrageenan-inducible genes). In cluster A, we identified pro-inflammatory cytokines and pain-associated genes including *Tnf, prostaglandin-endoperoxide synthase 2* (*Ptgs2*), and *gamma-aminobutyric acid* (*GABA-A*) *receptor, subunit alpha 6* (*Gabra6*). Cluster B was represented the upregulated genes in both groups, but more upregulated in Stat4 -/- mice than wild-type mice (Stat4-regulated carrageenan-inducible genes). In this cluster, we also identified anti-inflammatory cytokines and pain-associated genes (*Il4* and *Il10ra*). Cluster C showed genes downregulated in wild type mice but not changed in Stat4 -/- mice (wild type-specific carrageenan-suppressed genes), including *opioid receptor, sigma 1* (*Oprs1*) and *gamma-aminobutyric acid* (*GABA-B*) *receptor, 1* (*Gabbr6*). Finally cluster D were genes downregulated in Stat4 -/- mice but not changed in wild type mice (Stat4-specific carrageenan-suppressed genes), including *Il6* and *Tnf receptor-associated factor 2* (*Traf2*).

Discussion

Acute inflammatory pain is characterized by local injury and inflammation, and can be regulated by various endogenous molecules. Pro-inflammatory and anti-inflammatory cytokines play important roles in acute inflammatory pain. STAT4 is transcription factor that mainly mediate IL12 signaling in inflammatory response. Recent findings consistently were demonstrated that IL12 induced pain in humans. Gollob *et al.*[3] reported that intravenous rhIL12 therapy for metastatic renal cancer or malignant melanoma patients was revealed arthralgias involving primarily the shoulders and fingers. Lenzi *et al.*[15] demonstrated that patients with intraperitoneally injected rhIL12 treatment for Müllerian carcinoma, gastrointestinal primary malignancies, and mesothelioma had headache and abdominal pain. Bladder spasms and pain were adverse effects related to the intravesicular treatment with rhIL12 for bladder carcinoma[16]. Mild to moderate pain at the site of injection has been reported in patients that received peritumoral injection of IL12-induced autologous fibroblasts[17]. However, the exact mechanisms underlying IL12-induced pain have not yet been investigated. Therefore, in the present study, we investigated the possible hypoalgesic effect of Stat4 -/-. Here, we reported that Stat4 -/- mice reduced paw volume in carrageenan-induced acute inflammation, and changed expression of several genes in brain. In the cluster A analysis, *Tnf, Ptgs2*, and *Gabra6*, were found to be upregulated in wild-type mice compared with Stat4 -/- mice (Table 1). Previous study was reported that Tnf and prostaglandin (PG) had hyperalgesia effect in inflammatory pain[7,8]. These results indicated that Tnf and Ptgs2 might be involved in reduced inflammatory responses in Stat4 -/- mice. Also, expression of some anti-inflammatory cytokines (*Il10ra* and *Il4*) was markedly higher in Stat4 -/- mice (Table 1). Cunha *et al.*[18] reported that Il4 released by mast cells limits inflammatory hyperalgesia, through inhibition of the production Tnf, Il1b, Il8, and PGs. Previous study was suggested that Il10 reduces the inflammatory hyperalgesia induced by carrageenan and bradykinin by two mechanisms; inhibition of cytokine production, inhibition of Il1b evoked PGE2 production[19]. We also funded that *Oprs1* and *Gabbr1* were downregulated in wild type, while pro-inflammatory associated genes such as *Il6* and *Traf2* were downregulated in Stat4 -/- mice (Table 1). Our results indicate that STAT4 might be related inflammatory response in the periphery and changed pain pathway in central system. Therefore, IL12-STAT4 signal pathway could be involved in carrageenan induced inflammation.

Table 1. Clustering analysis of brain gene expression.

Symbol	Gene name	Fold	Accession number
Cluster A			
Ndufs2	NADH dehydrogenase (ubiquinone) Fe-S protein 2	2.5	AW214481
Tnf	Tumor necrosis factor alpha	2.5	M13049
Tnfaip8	Tumor necrosis factor, alpha-induced protein 8	2.8	AI839109
Ptgs2	Prostaglandin-endoperoxide synthase 2	2.4	M94967
Mgl2	Macrophage galactose N-acetyl-galactosamine specific lectin 2	3.1	AI480756
Gabra6	Gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 6	2.8	AI839865
Cluster B			
Bmi1	B lymphoma Mo-MLV insertion region 1	2.6	AI852579
Tde2	Tumor differentially expressed 2	2.3	AI854679
Ramp2	RIKEN cDNA 9430072K23 gene	2.2	AI119013
Il10ra	Interleukin 10 receptor, alpha	2.2	AI173487
Max	Max protein	1.8	AI836400
Il4	Interleukin 4	2.3	AI528678
Mbnl1	Muscleblind-like 1 (Drosophila)	2.5	AI854176
Atp6v0c	ATPase, H+ transporting, V0 subunit C	1.9	AI385732
Sh3kbp1	Sh3kbp1 binding protein 1	3.1	AI482175
Abcc9	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	2.9	AI482206
Atp6v1a1	ATPase, H+ transporting, V1 subunit A, isoform 1	2.0	AI503947
Scd2	Stearoyl-Coenzyme A desaturase 2	2.0	BC040384
Rtn3	Reticulon 3	2.7 2.0	AI854888
Rnf13	Ring finger protein 137	2.6	AI894158
Bnip2	BCL2/adenovirus E1B 19 kDa-interacting protein 1, NIP2	2.1	AW107996
Cluster C			
Oprs1	Opioid receptor, sigma 1	−2.1	AI504954
Apc1	Anaphase promoting complex subunit 1	−2.7	AI893639
Ptprk	Protein tyrosine phosphatase, receptor type, K	−2.7	AI893646
Crsp8	Cofactor required for Sp1 transcriptional activation, subunit 8	−2.8	AI852972
Ak1	Adenylate kinase 1	−2.6	AI853614
Abr	Active BCR-related gene	−2.5	AI594620
Btn1a1	Butyrophilin, subfamily 1, member A1	−2.3	AI606486
Gabbr1	Gamma-aminobutyric acid (GABA-B) receptor, 1	−2.0	AI853724
Pfkfb3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	−3.0	AI326331
Etv4	Ets variant gene 4 (E1A enhancer binding protein, E1AF)	−2.2	AA959284
Dll1	Hypothetical protein A930024N18	−3.1	AI323450
Cluster D			
Plxnb1	Plexin B1	−1.9	AI504330
LOC330189	Similar to transmembrane protein induced by tumor necrosis factor alpha	−1.4	AI427441
Cox6a1	Cytochrome c oxidase, subunit VI a, polypeptide 1	−1.5	AI893442
Sprr2a	Small proline-rich protein 2B	−1.7	AI414574
Golga4	Golgi autoantigen, golgin subfamily a, 4	−1.6	AA647230
Triobp	TRIO and F-actin binding protein	−2.0	AI552425
Mkks	McKusick-Kaufman syndrome protein	−1.9	AI847618
Traf2	Tnf receptor-associated factor 2	−3.1	AI552593
Ndufa2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2	−1.7	AI326170
Basp1	Brain abundant, membrane attached signal protein 1	−3.0	AI894132
Metapl1	Methionine aminopeptidase-like 1	−2.7	AI326875
Taldo1	Transaldolase 1	−2.0	AI844299
Il6	Interleukin 6	−2.4	NM_031168

Methods

Animals

Adult male Stat4 -/- on a BALB/c background were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed in pairs at standard temperature (22 ± 3°C) and under a standard 12 h light/dark cycle with free access to food and water. All procedures involving animals and their care con-

formed to the international guidelines set out in principles of Laboratory Animal Care (NIH publication no.85-23, revised 1995).

Carrageenan Induced Paw Edema

Acute inflammation was induced in the right hind paw of mouse by subcutaneous injection with a 30 μL suspension of 1% freshly prepared λ carrageenan (Sigma, Seoul, Korea) in saline. Saline was injected to the control group. Paw edema was measured with a hydroplethysmometer (Ugo Basile 7140, Plethysmometer, Varese, Italy) at 0, 1, 2, 3, 4, and 5 h after the injection. The percent increase in paw volume (edema volume) was calculated by subtracting the paw volume measured prior to carrageenan injection from the paw volume measured after the edema was induced.

Analysis of cDNA Microarray

The Twinchip Mouse-7.4 k cDNA microarray (Digital Genomics, Seoul, Korea) was used. In brief, the cDNA synthesis was performed with 3DNA™ Array 50™ detection method (Genisphere, Hatfield, PA, USA) as per the manufacturer's protocols. Single-stranded cDNA probes were purified using a PCR purification kit (Qiagen, Seoul, Korea). Probes were resuspended in hybridization solution containing 50% formanide, 5 × SSC, 0.1% SDS. The fluorescent-labeled cDNA were mixed and hybridized with The Twinchip Mouse-7.4 k cDNA microarray at 42°C in a humid chamber. The hybridized microarray was scanned with a confocal laser scanning microscope (ScanArray 5000; Packard Inc., Billerica, MA, USA). Scanned image were analyzed with GenePix (Axon Inc., Sunnyvale, CA, USA) produced quantitative values for each microarray spot. Data normalization was performed by intensity/location-dependent method. Normalized spot intensities were calculated into gene expression ratios between the control and treatment groups. Mean data acquired from two identical arrays in a single slide of Twin Chip™ were analyzed.

Cluster Analysis, Data Annotation

Tree view software was applied for cluster analysis of detected genes. Genes with at least two fold expression values were included in the cluster analysis. Functional category classification was based on the National Center for Biotechnology Information Locus Link (http://www.ncbi.nlm.nih) and Gene Ontology (http://www.geneontology.org) databases, which classify a gene according to molecular function, biological process, and cellular component.

Statistical Analysis

Values were expressed as the means standard error of the means (S.E.M.). The results were analyzed by Mann-Whitney *U*-test for comparison of between STAT4 deficient and wild type groups. *P*-values less than 0.05 were considered significant.

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