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cDNA Microarray Analysis of the Differential Gene Expression in the Neuropathic Pain and Electroacupuncture Treatment Models

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Partial nerve injury is the main cause of neuropathic pain disorders in humans. Acupuncture has long been used to relieve pain. It is known to relieve pain by controlling the activities of the autonomic nervous system. Although the mechanism of neuropathic pain and analgesic effects of electroacupuncture (EA) have been studied in a rat model system, its detailed mechanism at the molecular level remains unclear. To identify genes that might serve as either markers or explain these distinct biological functions, a cDNA microarray analysis was used to compare the expression of 8,400 genes among three sample groups. Messenger RNAs that were pooled from the spinal nerves of 7 normal, 7 neuropathic pain, and 7 EA treatment rat models were compared. Sixty-eight genes were differentially expressed more than 2-fold in the neuropathic rat model when compared to the normal, and restored to the normal expression level after the EA treatment. These genes are involved in a number of biological processes, including the signal transduction, gene expression, and nociceptive pathways. Confirmation of the differential gene expression was performed by a dot-blot analysis. Dot-blotting results showed that the opioid receptor sigma was among those genes. This indicates that opioid-signaling events are involved in neuropathic pain and the analgesic effects of EA. The potential application of these data include the identification and characterization of signaling pathways that are involved in the EA treatment, studies on the role of the opioid receptor in neuropathic pain, and further exploration on the role of selected identified genes in animal models.

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Introduction

Neuropathic pain is part of the neurological disease spectrum and may be an expression of severe medical pathology (Hansson, 2002). Partial nerve injury is the main cause of neuropathic pain disorders in humans. The pain is characterized by a spontaneous burning pain that is accompanied by hyperalgesia and allodynia that lasts variable time spans (Talmoush, 1981). As already observed, this pain is often unresponsive to analgesics. Acupuncture has long been used to relieve pain. It is known to relieve pain by controlling the activities of the autonomic nervous system (Kimura and Sato, 1997). The descending inhibitory system and endogenous opioid (<biblio>) mediate this pain relief function of acupuncture (Han and Terenius, 1982). It has also been proposed that acupuncture triggers diffuse noxious inhibitory control on convergent neurons at the medullary and spinal levels (Bing *et al*., 1990).

Although the mechanism of neuropathic pain and analgesic effects of electroacupuncture (EA) have been studied in a rat model system, its detailed mechanism at the molecular level remains unclear. As a marker of neuronal activation of the central nervous system, the cellular fos (c-fos) expression has been widely used to monitor the change in neuronal activity that is evoked by peripheral input (Doucet *et al.*, 1990; Ji *et al.* 1993; Guo *et al*., 1996). C-fos proto-oncogene is an immediate early-response (IER) gene, which is characterized by the rapid, stimulus - mediated induction of the expression in neuronal and non-neuronal cells (Doucet *et al.*, 1990; Bogoyevitch *et al*., 2001). Besides IER genes, several opioid genes including preproenkephalin (PPE), preprodynorphine (PPD) and proopiomelanocortin (POMC) are expressed by the

EA treatment (Lee and Beitz, 1992; Pan *et al.*, 1994; Ji *et al.*, 1993; Guo *et al.*, 1996). Although evidence from recent studies indicate that several genes are involved in neuronal activities, little information is available regarding the genes that are involved in the development of neuropathic pain and the analgesic effects of EA.

In the present study, we developed a neuropathic animal model that showed causalgic signs on the tail. We analyzed the genes that were expressed differentially in the neuropathic pain model and EA treatment model using cDNA miroarray.

Materials and Methods

Experimental animals Young adult male Sprague-Dawley rats (Sam : TacN(SD)BR, 200-220 g, n = 21) were housed in group cages (4-5 per cage) with water and food available *ad libitum*. The room was light/dark (08 : 00 - 20 : 00 light, 20 : 00 - 08 : 00 dark) controlled and kept at 21-24°C. All of the experiments were conducted in accordance with the guidelines of the International Association for the Study of Pain.

Neuropathic surgery For neuropathic surgery, the rat-tail model was used, as described previously (Na *et al.*, 1994; Kim *et al.*, 2001). Briefly, under sodium pentobarbital anesthesia (40 mg/kg, i.p.), the right superior caudal trunk was exposed and transected at the level between the S1 and S2 spinal nerves that innervated the rat tail. To prevent the possible rejoining of the proximal and distal ends of the severed trunk, about a 2 mm piece of the trunk was removed from the proximal end. This surgery eliminated the S1 spinal nerve innervation to the tail via the right superior caudal trunk.

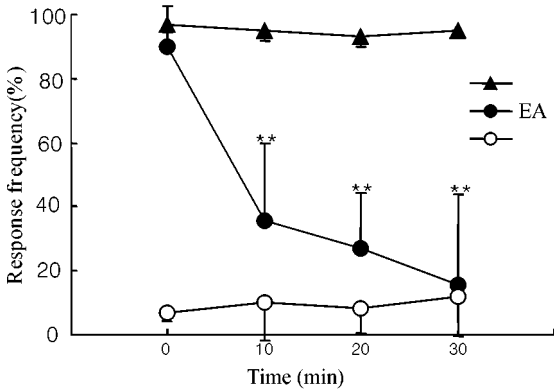

Fig. 1. Pain relief effect of EA treatment on mechanical allodynia. Three weeks after the neuropathic surgery, the control (n = 7) and EA treatment groups (n = 7) showed a high response frequency. The EA treatment group was given train-pulse (2 Hz, 0.3 ms pulse-width, 0.2-0.3 mA) for 30 min. In the EA treatment group, there were marked decreases in response frequency, whereas the control and normal sham-operation groups (n = 7) showed no significant changes. Asterisks indicate the values that were significantly different from the value before EA treatment (p<0.01 by the Friedman rank test followed by the Dunnett post-hoc test).

Behavioral test of neuropathic pain The mechanical allodynia was assessed by a normally innocuous stimulation of the tail with von Frey hair (bending force: 2.0 g). For a convenient application of stimuli with von Frey hair and acupuncture, the rat was restrained in a plastic holder (5.3×15, 6.3×18 cm in diameter $\times$ length), and the tail was laid on a plate. The mechanically-sensitive area was first determined by rubbing various areas of the tail with von Frey hair. The actual test was performed by gently poking the most sensitive spot with von Frey hair. An abrupt tail movement of more than 0.1 cm was considered an abnormal response that was attributed to mechanical allodynia. The stimulation was repeated 10 times at 10-sec intervals for each animal at each testing time or day. During the repeated trials, the test stimuli were delivered to the same spot with no difficulty, since the tail was usually stationary. The degree of response was expressed as a percentage of response frequency and was determined as follows;

$$\text{Response Frequency (\%)} = \frac{\text{number of abnormal responses}}{10} \times 100$$

EA stimulation Two stainless-steel needles of 0.25 mm diameter and 5 mm in length were inserted into Zusanli (ST36), which is located at the anterior tibial muscle and about 10 mm below the knee joint. This point produces an analgesic effect in the tail-flick response. For EA, train-pulses (2 Hz, 0.3-ms pulse width, 0.2-0.3 mA) were applied to the inserted needle for 30 min. The other needle (anode) was inserted into the anterior tibial muscle 5 mm that was distal to the first one. Anodal and cathodal leads from an electric stimulator were connected to the two acupuncture needles.

Table 1. Overall summary of the differential gene expression profile between normal and neuropathic pain models. Spinal cord mRNA was obtained from a normal rat sample**,** neuropathic pain model. The expression level intensity was determined using image analyzing software.

	Total genes expressed differentially: 11 Genes	
	> 2 fold increased	> 2 fold decreased
Known	5	5
Unknown	0	1
Total	5	6

Table 2. Overall summary of the differential gene expression profile between the neuropathic pain and EA treatment models. Spinal cord mRNA was obtained from the neuropathic pain and EA treatment models. The expression level intensity was determined using image analyzing software.

	Total genes expressed differentially: 45 Genes	
	> 2 fold increased	> 2 fold decreased
Known	6	34
Unknown	2	3
Total	8	37

Table 3. Genes decreased in the neuropathic pain model and were restored to the normal expression level after EA treatment

	Gene Bank ID	Gene Name
1	NM_011014	opioid receptor sigma 1
2	XM_001491	fucose-1-phosphate guanylyltransferase
3	BE388188	membrane-bound transcription factor protease, site 1
4	H99890	TAR (HIV) RNA-binding protein 1
5	AI051561	Homo sapiens cDNA FLJ13706 fis, clone PLACE2000317
6	AA436249	ESTs
7	AW627377	DKFZP434N093 protein
8	AK023010	chromosome condensation 1-like
9	AW241356	hypothetical protein FLJ10261
10	AA447415	15 kDa selenoprotein
11	Z98044	hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)
12	XM_013097	zinc finger protein 6 (CMPX1)
13	BF439351	Novel human gene mapping to chromosome 13
14	AI312130	chromosome 2 open reading frame 1
15	AW503100	UDP-glucose:glycoprotein glucosyltransferase 1
16	AI056157	ESTs
17	BF000639	Homo sapiens cDNA: FLJ22063 fis, clone HEP10326
18	S59049	regulator of G-protein signaling 1
19	AF196970	Wiskott-Aldrich syndrome (eczema-thrombocytopenia) Nck associated protein
20	BF055337	chromodomain protein, Y chromosome-like
21	AA494538	ESTs
22	C06042	carcinoembryonic antigen-related cell adhesion molecule 7
23	AI868427	95 kDa retinoblastoma protein binding protein; KIAA0661 gene product
24	AW770225	KIAA0996 protein
25	NM_004461	phenylalanine-tRNA synthetase-like
26	XM_003352	calmegin
27	AW847768	myosin phosphatase, target subunit 1
28	X12458	Protein P3
29	XM_002762	tumor necrosis factor, alpha-induced protein 6
30	BG032225	integrin cytoplasmic domain-associated protein 1
31	BF001626	homeo box (expressed in ES cells) 1
32	BF476140	KIAA1018 protein
33	XM_005991	KIAA0092 gene product
34	U81504	adaptor-related protein complex 3, beta 1 subunit
35	XM_005135	GATA-binding protein 4
36	N24973	electron-transfer-flavoprotein, alpha polypeptide (glutaric aciduria II)
37	AI816983	ESTs, Weakly similar to unnamed protein product [H.sapiens]
38	X06557	T cell receptor delta locus
39	AA927462	ESTs
40	AA894779	TATA-binding protein-binding protein
41	AF067420	VPS28 protein
42	AB011128	KIAA0556 protein
43	XM_013068	diaphanous (Drosophila, homolog) 2
44	AI870850	RAD1 (S. pombe) homolog
45	AW167014	hypothetical protein IMPACT
46	BF058544	DnaJ (Hsp40) homolog, subfamily C, member 4
47	AA972074	ESTs
48	AI394426	mitogen-activated protein kinase 11
49	BE745439	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 3
50	AA442098	hypothetical protein dJ551D2.5
51	AI718055	growth arrest and DNA-damage-inducible, alpha

Table 3. Continued

	Gene Bank ID	Gene Name
52	AA507382	ESTs
53	BE856915	Homo sapiens cDNA: FLJ23546 fis, clone LNG08361
54	BF967869	tetraspan 3
55	AI110705	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit (oligomycin sensitivity conferring protein)
56	N70239	KIAA0721 protein
57	AI499593	iroquois homeobox protein 5
58	BF515939	GL002 protein
59	AA417878	ESTs, Weakly similar to ALU8_HUMAN ALU SUBFAMILY SX SEQUENCE CONTAMINATION WARNING ENTRY [H.sapiens]
60	U12128	protein tyrosine phosphatase, non-receptor type 13 (APO-1/CD95 (Fas)-associated phosphatase)
61	AI572906	stromal antigen 3
62	AA811893	Homo sapiens mRNA; cDNA DKFZp761G1111 (from clone DKFZp761G1111)
63	AA455079	ESTs
64	AI168277	ESTs
65	AA196322	LIM protein (similar to rat protein kinase C-binding enigma)
66	AU139950	regulator of mitotic spindle assembly 1
67	AA905765	ESTs
68	AL136561	Homo sapiens mRNA; cDNA DKFZp761D221 (from clone DKFZp761D221); complete cds

Preparation of Tissues After 30 min of daily EA stimulation, as well as behavioral tests, the rat was sacrificed under anesthesia. The skull was then immediately opened. The brain was removed and dissected. For spinal cord isolation, a laminectomy was performed. The spinal cord at the level between S1 and S2 was carefully removed, then frozen immediately in liquid nitrogen, and kept at −70°C.

cDNA Microarray Analysis Gene Discovery Array Mouse version 1.1 (Incyte genomics, Palo Alto, CA) was used for a cDNA microarray analysis. It consisted of one 22 × 22 cm nylon filter that was spotted with 8,400 non-redundant mouse cDNA clones that was chosen from the I.M.A.G.E. collection. Total RNAs were isolated from the spinal cord using Trizol (Life Technologies, Gaithersburg, MD), followed by the isolation of mRNA using OligoTex (Qiagen, Valencia, CA). The cDNA preparation was labeled with [α-^{32}P]dCTP and hybridization was performed according to the manufacturers instruction. After hybridization, the filters were exposed to X-ray film and quantified by scanning densitometry.

Dot Blot Analysis Total RNAs were isolated from the spinal cord using RNA Stat-60 (TEL-TEST, Inc., Friendswood, TX) according to the manufacturers instruction. Two μg of total RNA was denatured and dotted onto a Hybond N^+ nylon membrane (Amersham Pharmacia Biotech., Piscataway, NJ). Cross-linking was then performed using UV irradiation. The filters were prehybridized overnight at 42°C in a Northern-Max hybridization solution (Ambion, Inc., Austin, TX). Hybridization was carried out overnight at 42°C in the same solution that contained probes that were labeled with [α-^{32}P]dCTP (DuPont NEN, 6000 Ci/mmol) by the random primer method. The DNA probes were synthesized by a reverse transcriptase-polymerase chain reaction method using rat brain-derived mRNA as a template. The following probes were used in these experiments: (1) a 445-bp cDNA fragment corresponding to bases 777-1221 of the reported opioid receptor

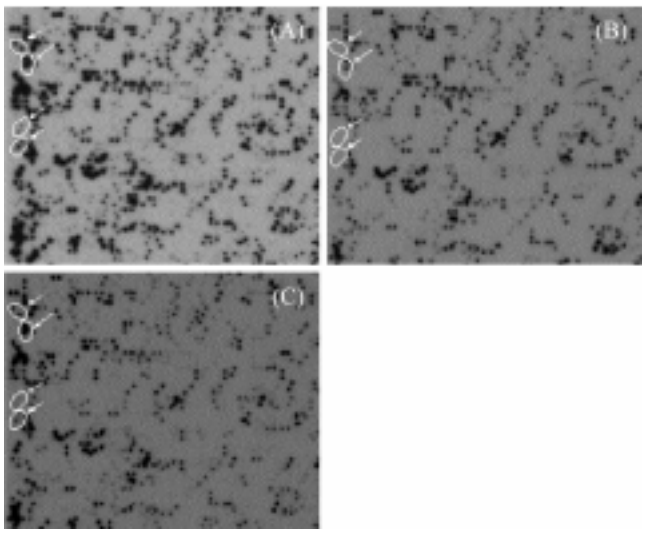

Fig. 2. Expression profile of normal (A), neuropathic pain (B), and EA treatment models (C). Spinal cord mRNA was obtained from a normal rat sample, neuropathic pain model that was developed previously (Kim *et al*., 2001), and EA treatment sample that was stimulated with EA for 30 min a day for 2 days. The expression level intensity was determined using image analyzing software. Arrows indicate examples of the differentially-expressed genes.

sigma (left primer: GCAGGACCACATCCATCTCT, right primer: TGAAGGGAAGGGCATCATAG); (2) a 401-bp cDNA fragment corresponding to bases 297-697 of the reported MAPK11 (left primer: CAGCGAAGTGTACCTCGTGA, right primer: TCAGCT GGTCGATGTAGTCG); (3) a 400-bp cDNA fragment corresponding to bases 4823-5222 of the reported APO-1/CD95 (Fas)-associated phosphatase (left primer: TTGACCCCACTTCAG TCTCC, right primer: GAGCCATATCCGGTGGTAGA); (4) a 397-bp cDNA fragment corresponding to bases 187-583 of the reported LIM protein (left primer: GACAGCACAACAGTGGCA AT, right primer: GCCCAAAGTTCTTTGCGTAG); (5) a 404-bp cDNA fragment corresponding to bases 1619-2022 of the reported Nck-associated protein (left primer: TCCACACTCACTCTGCC ATC, right primer: GGCTGTCCTGGAACTCACTC); (6) a 399-bp cDNA fragment corresponding to bases 1203-1601 of the reported iroquois homeobox protein 5 (left primer: ATCAGGAGGAGCAG AGCAGA, right primer: TTGACCTTGTCCGAGGATGT); (7) a 399-bp cDNA fragment corresponding to bases 924-1322 of the reported tumor necrosis factor α-induced protein 6 (left primer: AGTGATGGAGATCCCTGTGC, right primer: CTCCAGTTTGA GGAGCCAAG); (8) a 402-bp cDNA fragment corresponding to bases 3098-3499 of the reported diaphanous 2 (left primer: TGTTGCATTTCTTGGCTGAG, right primer: GGTTTTCCTTGA CTGCTTGC); (9) a *Xba*I-*Hind*III-digested 500-bp fragment of glyceraldehydephosphate dehydrogenase from the pUC/GAPDH. After hybridization, the filters were washed with 2 × SSC/0.1% SDS for 20 min at room temperature, 2 × SSC/0.1% SDS at 42°C for 30 min, and 0.5 × SSC/0.1% SDS for 30 min at 52°C.

Data analysis Data were represented as means ± standard error. The significance of the statistical differences was determined using the Friedman rank test, followed by the Dunnett post-hoc test in a group. $p < 0.05$ was considered significant.

Results and Discussion

Pain relief effect of EA on mechanical allodynia Recently, several behavioral models for peripheral neuropathy that were produced in a rats tail were developed. We used a previously-developed rat model for neuropathic pain (Na *et al*., 1994; Kim *et al*., 2001). The sign of mechanical allodynia appeared 1 day after surgery; maximal allodynia was observed in 2-3 weeks (Hwang *et al*., 2002). Following this result, EA was applied three weeks after surgery. Behavioral tests of mechanical allodynia were performed before and during EA at 10-min interval. In the EA-treatment group (n = 7), there were marked decreases in the response frequency (90.014.1, 35.724.4, 27.118.0 and 15.728.8%, respectively). The Dunnett post-hoc test, after the Friedman rank test, indicated that there

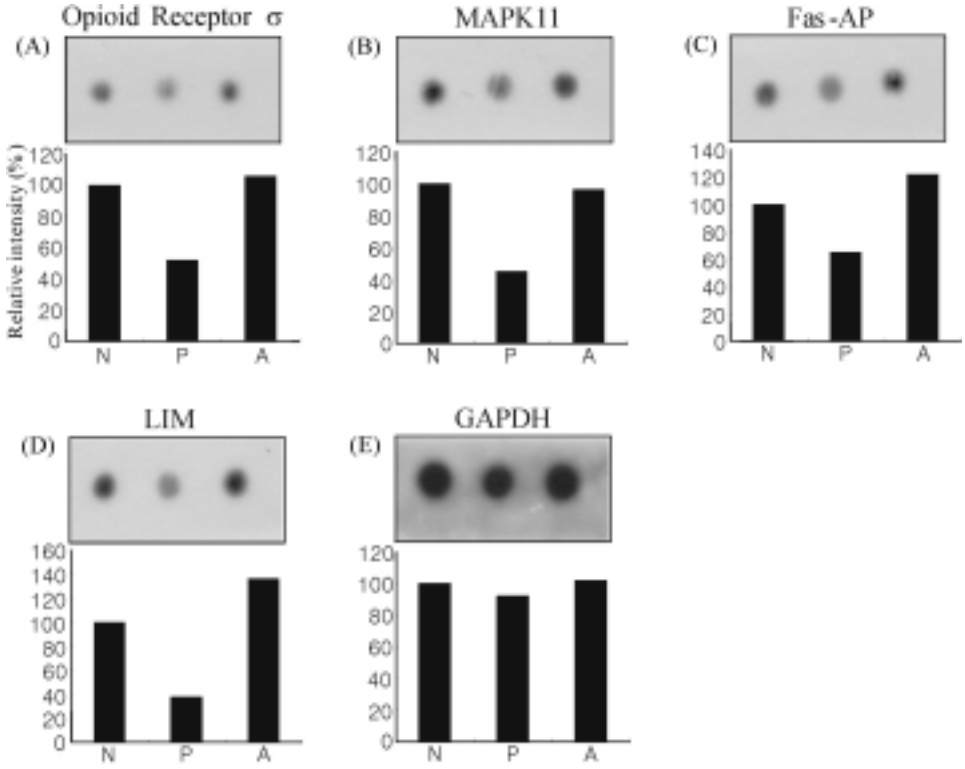

Fig. 3. Genes involved in signal transduction are expressed differentially in the pain model and restored after EA treatment. Total RNAs (2 μg) were isolated from the spinal cord that was obtained from the normal (N), neuropathic pain (P), and EA treatment models (A). After the total RNAs were dotted on nylon filters, the blots were hybridized with ^{32}P-labeled probes. The probes that were used in the dot-blot analysis were opioid receptor sigma (A), MAP kinase 11 (B), APO-1/CD95 (Fas)-associated phosphatase (C), and LIM protein (D). GAPDH (E) total RNAs were used as a control.

were statistically significant differences at 10, 20, and 30 min from the initiation of EA. The maximal pain relief effect of EA on mechanical allodynia was shown at 30 min after the initiation of EA (Fig. 1).

cDNA microarray analysis To identify genes that might either serve as markers or explain neuropathic pain development, or the analgesic effects of EA, a cDNA microarray analysis was used to compare the expression of 8,400 genes among three sample groups. Messenger RNAs were compared that were pooled from the spinal nerves of 7 normal, 7 neuropathic pain, and 7 EA treatment rat models. Eleven genes were identified as differentially-expressed genes between the normal and neuropathic pain models (Table 1). Among these 11 genes, 5 genes increased and 6 genes decreased in the pain model. These genes were involved in the transcription, protein synthesis, and degradation process (data not shown). Table 2 shows that the gene numbers were expressed differentially between the neuropathic pain and EA treatment models. Among 45 genes, 8 genes increased and 37 genes decreased in the EA treatment rat model, compared to the neuropathic pain rat model. These genes include receptors, signaling molecules, protein kinases, and chaperonic proteins (data not shown).

Since acupuncture treatment is known to relieve neuropathic pain, we focused on the genes that were differentially expressed in the pain model when compared to the normal, and restored to the normal expression level after the EA treatment. As shown in Table 3, 68 genes were differentially expressed more than 2-fold in the neuropathic rat model when compared to the normal, and restored to the normal expression level after the EA treatment. These genes are involved in a number of biological processes, including signal transduction, gene expression, and nociceptive pathway. The genes that were involved in the signal transduction pathway include the mitogen-activated protein (MAP) kinase (Kim *et al.*), protein tyrosine phosphatase, protein kinase c binding protein, regulator protein of the G-protein signaling, and T cell receptor. The genes that were involved in the gene expression regulation include the zinc finger protein 6 and TATA-binding protein-binding protein. Interestingly, the opioid receptor sigma was one of those genes that was expressed differentially in the neuropathic pain model, and restored to the normal level after the EA treatment (Table 3). However, one third of the genes that were identified were unknown genes, or functionally uncharacterized genes.

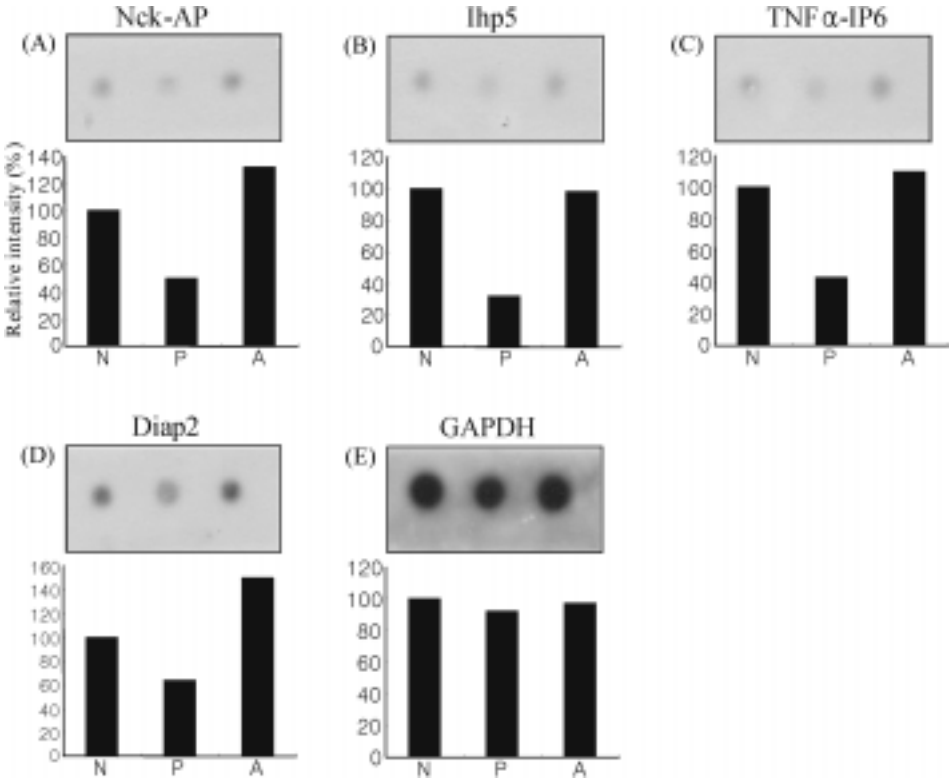

Fig. 4. The genes that were involved in the various biological processes are expressed differentially in the pain model and restored after EA treatment. Total RNAs (2 μg) were isolated from the spinal cord that was obtained from normal (N), neuropathic pain (P), and EA treatment models (A). After the total RNAs were dotted on nylon filters, the blots were hybridized with ^{32}P-labeled probes. The probes that were used in the dot-blot analysis were Nck-associated protein (A), iroquois homeobox protein 5 (B), tumor necrosis factor α-induced protein 6 (C), and diaphanous 2 (D). GAPDH (E) total RNAs were used as a control.

Opioid receptor is involved in analgesic process of EA Confirmation of the differential gene expression was performed by a dot-blot analysis. Among 68 genes that we considered, the mRNA expression level of 8 genes was down-regulated in the neuropathic pain model when compared to the normal rat, and the mRNA expression level of these genes was restored to the normal state in the EA treatment sample. Results from dot blotting showed that the opioid receptor sigma was one of those genes, indicating that the opioid signaling event is involved in neuropathic pain and the analgesic effects of EA. The expression level of the opioid receptor sigma gene decreased about 50% in the neuropathic pain model. However, 1 day after the EA treatment, the mRNA expression level of the opioid receptor sigma was restored to its normal expression level. This result indicates that the cells in the spinal cord cannot normally transduce the signal in response to opioid stimulation since the opioid receptor is down-regulated in the neuropathic pain model. However, the EA treatment restores the expression level of the opioid receptor in response to the opioid signal that gives rise to analgesic effects. It was reported that endogenous opioids may be implicated in acupuncture analgesia (He, 1987). For example, acupuncture produces an increase in the opioid-like immunoreactivity in the CSF of patients with chronic pain from different sources (Ho and Wen, 1989). Our data suggest that the opioid receptor probably plays an important role in the development of neuropathic pain and the analgesic effects of EA.

Besides the opioid receptor, several interesting genes, such as the MAP kinase, zinc finger protein, and tyrosine phosphatase, were identified. The expression level of these genes decreased about 40-60% in the neuropathic pain model. However, 1 day after the EA treatment, the mRNA expression level of these genes was restored to the normal expression level. These genes are known to function on important signal transduction pathways and gene expressions. Therefore, multiple signaling pathways, including opioid receptor- and MAP kinase-mediated pathways, as well as other gene expressions, might be involved in the pain development and analgesic effects of EA.

While further studies are required in order to delineate the signaling pathways that lead to chronic neuropathic pain and pain relief by acupuncture, this is the first report that analyzes the genes that are expressed differentially in the neuropathic pain and EA treatment models. From this information, we can begin to understand the molecular mechanism of pain development and analgesic effects of acupuncture, which are important events in pathological processes. The potential application of these data include the identification and characterization of signaling pathways that are involved in acupuncture treatment, studies on the role of the opioid receptor in neuropathic pain, and further exploration on the role of selected identified genes in animal models.

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