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Original Article

Role of TLR4/NF-κB signal transduction pathway in the formation of bone cancer pain in rats

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Abstract: Background: To investigate the role of Toll-like receptor 4 (TLR4)/NF-κB signal transduction pathway in formation of bone cancer pain (BCP) in rats. Methods: The experiments were performed using 81 unmated female SD rats, weighing 150-170 g and 5-7 weeks old. The rats were randomly assigned to eight groups. Group 1 (n=9) received nothing and served as control group. Group 2 (n=18) received intra-tibial injection of 1×10^5 Walker 256 breast cancer cells and served as BCP group. Group 3 (n=9), 4 (n=9), 5 (n=9) received menstruum,TLR4 small interfering RNA (TLR4siRNA) and TLR4 missense siRNA (TLR4msiRNA) intrathecal pre-injection on day 4 (pre-injection) after successful animal model, and group 6 (n=9), 7 (n=9), 8 (n=9) received above on day 9 (post-injection) respectively. The injections were given daily for three consecutive days, the rats were euthanized at 24-hour after the last injection for spinal cord of L_{4-6} lumbar segments examination. Expression of NF-κBp65 mRNA, IL-6 mRNA and TNF-α mRNA in spinal cord tissue were examined by PR-PCR, and expression of NF-κBp65 protein was analyzed by immunohistochemistry and Western blot technique. Results: Up-regulation of NF-κB65 mRNA, IL-6 mRNA and TNF-α mRNA in spinal cord tissues were completely inhibited after pre-injection of TLR4siRNA, however, they were partly inhibited after post-injection of TLR4siRNA ($P<0.05$). Conclusion TLR4/NF-κB signaling plays a pivotal role in intracellular signal transduction pathways involved in BCP states.

Keywords: Toll-like receptor 4, bone cancer pain, NF-kappa B, rat

Introduction

Bone cancer pain (BCP) associated with cancer metastasis is the most severe of all types of cancer pain, which frequently fails to response to the existing analgesics. However, its mechanisms is still unclear [1]. In comparison to neuropathic pain or inflammatory pain, BCP shows distinctly differences in the terms of cellular and neurochemical changes in the nervous system. To investigate its mechanism, it will help us develop more effective treatment for BCP.

Toll-like receptor 4 (TLR4) is a transmembrane receptor protein with certain extracellular leucine-rich repeat domains and a cytoplasmic signaling domain. It has been reported that TLR4 plays an important role in the development and maintenance of BCP in rats [2, 3]. Once activated, TLR4 can induce the synthesis of certain inflammatory cytokines through many signal transduction pathways, and it finally causes hyperalgesia. NF-κB signal transduction is one of the most important pathway, and many studies are focusing on this pathway recently [4].

Our previous study showed that NF-κB was associated with BCP in spinal cord of rat. In the present study, we we performed further research to investigate the role of TLR4/NF-κB signal transduction pathway in formation of BCP in rat and tried to determine the partial mechanism of BCP formation.

Materials and methods

Animals and grouping

The experiments were performed using 81 unmated female SD rats, weighing 150-170 g and aged 5-7 weeks old (from Experimental

Table 1. Expression of NF-κBp65 mRNA, IL-6 mRNA and TNF-α mRNA in all groups (n=3)

Group	NF-κBp65 mRNA	TNF-α mRNA	IL-6 mRNA
1	0.76±0.29	2.0±0.6	2.01±0.60
2			
Day 7	1.52±0.32[a]	2.9±0.4[a]	2.01±0.12
Day 12	3.66±1.03[a]	4.8±1.9[a]	7.03±1.05[a]
3	1.61±0.27[a]	2.8±1.3[a]	1.94±0.22
4	0.82±0.32[c]	2.1±0.7[c]	1.98±0.64
5	1.59±0.15[a]	3.0±0.4[a]	2.32±0.24
6	3.65±0.95[a]	4.7±1.1[a]	6.88±1.19
7	2.02±0.89[a,c]	3.8±1.5[a,b]	3.87±0.70[b,c]
8	3.55±1.21[a]	4.9±1.2[a]	7.01±1.63[a]

[a]P<0.05 vs. group 1; [b]P<0.05 [c]P<0.05 vs. group 2.

Animal Research Center of Shandong University). All experimental procedures and animal handling were performed according to both the Guiding Principles for the Care and Use of Laboratory Animals. The protocol was approved by the committee on the Ethics of Animal Experiments of the Shandong University. The rats accessed to food and water freely in a controlled temperature (22-24°C) and humidity (40-60%).

All rats were randomly assigned to eight groups. Group 1 (n=9) received nothing and served as control group. Group 2 (n=18) received intratibial injection of 1×10^5 Walker 256 breast cancer cells and served as BCP group. Group 3 (n=9), 4 (n=9), 5 (n=9) received menstruum (NM_13031A1S, Polyplus Company), TLR4siRNA and TLR4msiRNA intrathecal pre-injection on day 4 after successful animal modal, and group 6 (n=9), 7 (n=9), 8 (n=9) received above on day 9 respectively. TLR4siRNA and TLR4msiRNA 2 μg were diluted with 5% glucose and jetPEI™ to 5 μL, and injections were given daily for three consecutive days. The rats were euthanized at 24-hour after the last injection. In BCP group, the rats were euthanized on day 7 and 12 for spinal cord of L_{4-6} lumbar segments examination.

Preparation of BCP model

The BCP model was prepared according to reported literatures [5-7]. For the BCP model, 5 μL Walker 256 tumor cells were injected into the upper segment of left tibia except control group. The Walker 256 cells were maintained in Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum, 100 unit/ml penicillin, and 100 μg/mL streptomycin; and cultured at 37°C in a humidified atmosphere of 5% CO_2 then passaged weekly according to ATCC guidelines. For administration, cells were detached by scraping and then centrifuged at 900 rpm for 3 min. The pellet was suspended in Hank's balanced salt solution and then used for intra-tibial injection.

The implantation technique of the intrathecal catheter was modified and performed [8, 9]. A polyethylene-10 catheter was inserted under pentobarbital anesthesia (50 mg/kg). The catheter was passed 6.5 cm caudally through an incision and the external part of the catheter was tunneled subcutaneously. The skin was closed with 3-0 silk sutures. After surgery, rats were housed in individual cages. To avoid occlusion of the catheter, 10 μL of normal saline was injected via a catheter on alternate days until the end of the experiment. The marker of successful catheterization was that rats showed no impaired movement or lower limb paralysis within 30 s after 2% lidocaine (10 μL) was injected intrathecally.

PT-PCR analysis

Total RNA was extracted from spinal cord tissue of L_{4-6} lumbar segments in accordance with the TRIzol kit's instructions, and cDNA was amplified with 0.1 μg RNA according to reverse transcription-polymerase chain reaction kit's instructions. Primers were synthesized by Jierui Company (Shanghai, China) as follows: NF-κBp65 (92 bp), upstream 5'-GGTTTGAGACATCC CTGCTT-3', downstream 5'-TATGGCAGGTCTGG-TCTG-3'; IL-6 (129 bp), upstream 5'-AAG GACCAAGAC CATCCAAC-3', downstream 5'-AC-CACAGTGAGGAATGTCCA-3'; TNF-α (65 bp), upstream 5'-CTAACTCCCAGAAAAG CAAGCAA-3', downstream 5'-CCTCGGG CCAGTGTATGAGA-3'; β-actin (207 bp), upstream 5'-CACCCGCGAG-TACAACC TTC-3', downstream 5'-CCCATACCCA-CCATCACACC-3'. The amplification reaction involved 25 cycles of denaturation at 94°C for 20 s, annealing at 58°C for 20 s, and extending at 72°C for 1 min. Ratio of target gene band gray value to β-actin could reflect the gene expression level.

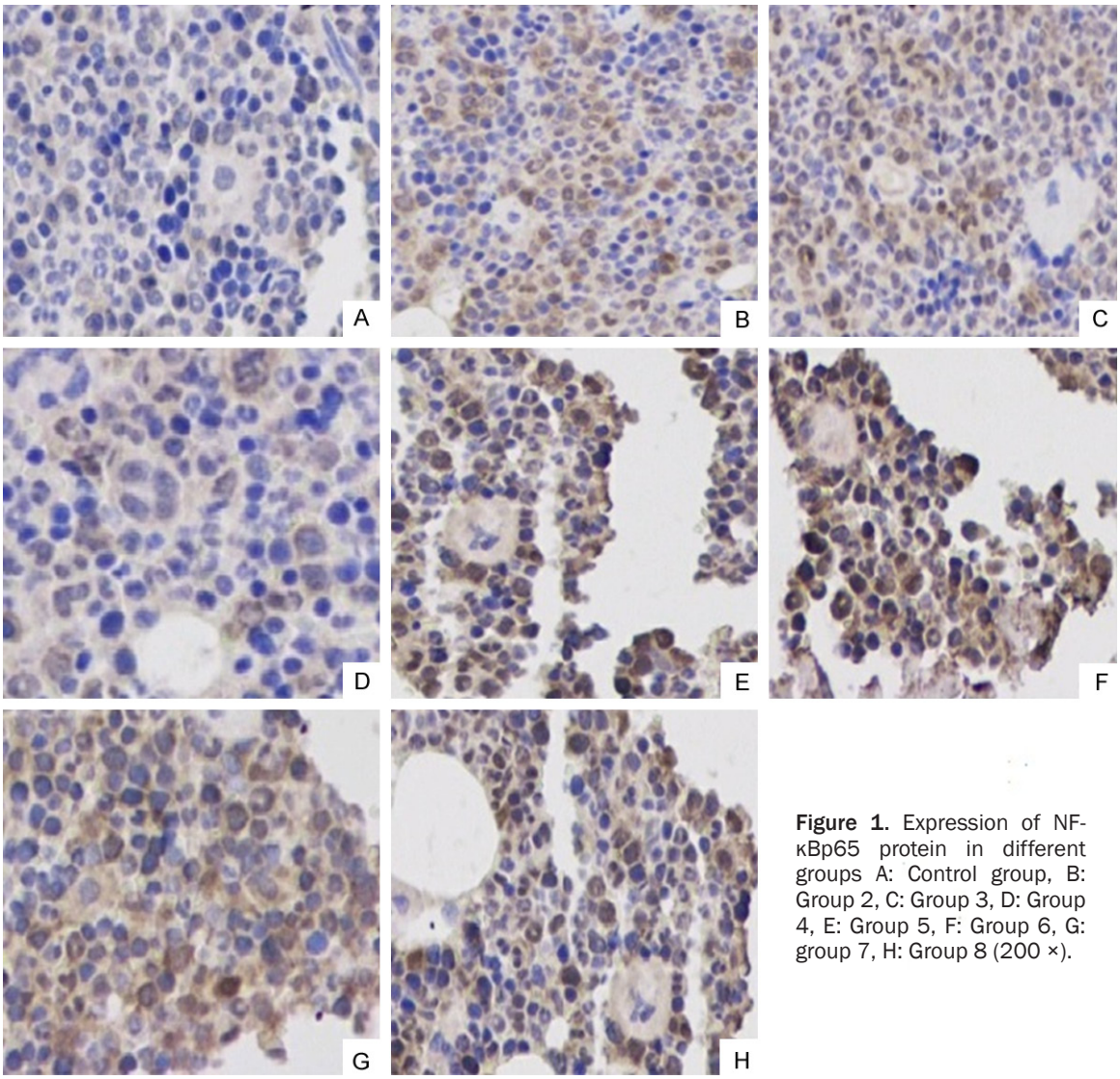

Figure 1. Expression of NF-κBp65 protein in different groups A: Control group, B: Group 2, C: Group 3, D: Group 4, E: Group 5, F: Group 6, G: group 7, H: Group 8 (200 ×).

NF-κBp65 protein expression by immunohistochemistry and Western blot

Paraffin-embedded tissue blocks were sectioned (4 μm-thick), dried, deparaffinized, and rehydrated. Antigen retrieval was performed in a microwave oven for 15 min in 10 mM citrate buffer (pH 6.0). For all samples, endogenous peroxidase activity was blocked with a 3% H_2O_2-methanol solution. The slides were blocked with 10% normal goat serum for 10 min and incubated with an appropriately diluted primary antibody rabbit anti-mouse NF-κBp65 antibody (1:100), and then goat anti-rabbit secondary antibody (1:1000) overnight at 4°C. The slides were then probed with an HRP-labeled polymer conjugated to an appropriate secondary antibody for 30 min. Each step was followed by washing with PBS. NF-κBp65 expression was examined under an inverted microscope. Five fields of cells were counted, respectively to determine whether the cells were positive for NF-κBp65 and analyzed with Image-pro Plus 6.0 system. Integral optical density (IOD) could reflect NF-κBp65 protein expression.

Cytoplasmic protein was extracted, and the protein concentration was determined using the Bradford method. The protein (100 μg) was added to each well for sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred to a polyvinylidence difluoride (PVDF) membrane for Ponceau red staining, the effect of a transfer film and the position of the target protein were determined according to the mark protein. The PVDF membrane was

Table 2. NF-κBp65 protein expression in all groups (n=3)

Group	Immunohistochemistry		Western blot
	IOD	Positive cells	
1	179±25	6.2±2.1	0.53±0.22
2			
Day 7	269±12[a]	29.4±7.7[a]	1.59±0.79[a]
Day 12	512±48[a]	46.8±11.6[a]	2.32±1.02[a]
3	322±42[a]	30.5±8.7[a]	1.61±0.88[a]
4	188±31[b]	7.2±2.4[b]	0.72±0.22[b]
5	292±32[a]	128.3±6.8[a]	1.62±0.61[a]
6	520±55[a]	47.5±12.5[a]	2.16±0.89[a]
7	288±52[a,b]	24.4±7.2[a,b]	1.41±0.52[a]
8	524±55[a]	48.2±10.5[a]	2.49±0.92[a]

[a]*P*<0.01 vs. group 1; [b]*P*<0.05 vs. group 2.

incubated with 5% bovine serum albumin at 37°C for 1 hour, followed by incubation with mouse anti-NF-κBp65 monoclonal antibody (1:100, Sigma Company) and then with horseradish peroxidase-labeled goat anti-mouse antibody (1:500, Sigma Company) at 37°C for 1 hour, respectively. β-actin was used as an internal reference. The signals were detected with an ECL chemiluminescence kit. The experiment in each group was repeated three times.

Statistics

Statistical analysis was done using SPSS 17.0 statistical software. Measurement data was expressed as mean ± standard deviation, then submitted for one-way ANOVA analysis. S-N-K (Student-Newman-Keuls) test as Post-hoc analysis was followed to compare among groups. P<0.05 was considered statistically significant.

Results

Expression of NF-κBp65 mRNA, IL-6 mRNA and TNF-α mRNA

NF-κBp65 mRNA and TNF-α mRNA expression in spinal cord tissue in group 2, 3, 5, 6, 7 and 8 were higher than that in control group (P<0.05). IL-6 mRNA expression up-regulated in group 2 (day 12), 6, 7 and 8 (P<0.05). NF-κBp65 mRNA and TNF-α mRNA expression in group 4 is low compared to that in group 2 (P<0.05), significant differences were not found among group 3, 5, 6 and 8 (P>0.05) (**Table 1**).

Expression of NF-κBp65 protein

Expression of NF-κBp65 protein in group 2, 3, 5, 6, 7 and 8 was higher than that in control group and group 4 (**Figure 1**) (P<0.05), however, there was no significantly different between group 4 and control group (P>0.05). NF-κBp65 expression was low in group 4 compared to group 2 (P<0.05), and differences were not found in group 3, 5, 6 and 8 were not found (P>0.05) (**Table 2**).

Discussion

Bone metastasis-associated pain is characterized by spontaneous pain, hyperalgesia, and allodynia [10]. Pain severe enough to compromise their daily lives affects 36%-50% of cancer patients [11]. To clarify the mechanisms of bone cancer pain, rat models of bone cancer pain using breast cancer cells (Walker 256 cell) have been established [13]. Our previous study showed that allodynia and spontaneous pain of rats' hindlimb could be seen on day 6 following inoculation with Walker 256 cells, and hyperalgesia strengthened with time. NF-κBp65 mRNA in spinal cord tissue increased on day 6 after inoculation and had a positive correlation with time. So in this study, tumor cells through intrathecal catheter were given on day 4 and 9.

RNA interference is that double stranded RNA with similar homologous region to endogenous mRNA degrades caused by its introduction into cells. Previous results also showed that transfection efficiency was the highest and effect of RNA interference was best when 40 nmol/L TLR4siRNA was added into microglia and cultured for 24 hours. So dose of TLR4siRNA and medication time were determined according to the literatures [13, 14] and pre-test results.

In the central nervous system, TLR4 is predominantly expressed by microglia. It has been proposed that the TLR4 is the key receptor in the formation of neuropathic pain without any exogenous lipopolysaccharide and exogenous pathogen [15]. Inoculation of tumor cells into tibial medullary cavity destroyed the bone and peripheral nerve, degraded fibrin of sensory nerve and sympathetic nerve. Lipopolysaccharide was produced during degradation which could activate TLR4. The activated TLR4 in the cells binds myeloid differentiation factor 88 (MvD88) receptor, and then binds to associated kinase of IL-1 receptor through MvD88 resulting to kinase autophosphorylation and the following activation of tumor necrosis factor receptor factor 6. NF-κB inhibitory protein family α, β kinase were activated by activating

NF-κB inhibiting protein kinase, resulting in NF-κB family extensive phosphorylation and degradation, followed by NF-κB translocation to the nucleus, transcription and translation of start cytokines of NF-κB active dimeric (IL-6, TNF-α) and co-stimulatory molecules CD80 and CD86. Ultimately, inflammatory cytokine synthesized increasingly which play an important role in regulation of chronic pain [16].

In this study, our results showed that antagonizing TLR4 could completely inhibit the activation of NF-κB and synthesis of TNF-α before formation of BCP, while activation of NF-κB and synthesis of TNF-α and IL-6 were partly inhibited during the formation of BCP. This finding suggested that TLR4 was the main mediator in the induction of BCP, and there is a potential role for other receptors to be involved in maintaining the pain state. These might include bradykinin, P2X3, TRPV1, and prostaglandin receptors, acid-sensing ion channel 3 and voltage-gated sodium channels, and unique glial/neuronal signals like fractalkine. Thus, our results underscore the complexity of central nervous system cascades and mediators that may underlie neuronal sensitization, the pathological manifestation of cancer pain [17].

In conclusion, our results provide evidence for a role of the key pathway of TLR4/NF-κB in a rat model of BCP. The ability of TLR4/NF-κB pathway leading to central sensitization and behavioral hypersensitivity could provide an opportunity for regulating glial activation, and thus alleviating BCP.

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Disclosure of conflict of interest

None.

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