Original Article

Inhibitory effects of intrathecal p38β antisense oligonucleotide on bone cancer pain in rats

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Abstract: Objective: To evaluate the effects of intrathecal administration p38β antisense oligonucleotide on the development of bone cancer pain rats. Methods: Forty female SD rats weighing 180~220 g were randomly divided into 4 groups (n = 10 each): Group A (control group): intra-tibial injection of 3 μl Hank’s solution; group B (model group): intra-tibial injection of 3 μl MADB-106 mammary gland carcinoma cells of rats (4.8 × 10³/μl); group C (p38β-SODN 20 μg); group D (p38β-ASODN 20 μg). The model procedures in group C and D were same to those in the group B. From the 14th day after operation, p38β-SODN 20 μg and p38β-ASODN 20 μg were respectively intrathecally administered in group C and D once daily for 6 days whereas normal saline was for group A and B. Mechanical withdrawal threshold and radiant heat threshold of rat hind paws were measured before operation and every other day until 22 d of post-operation. The lumbar 4-6 spinal cord was removed on the 22nd day. The expression of spinal p38β protein was determined by Western blot. Results: No significant differences in mechanical withdrawal threshold and radiant heat threshold were found at all time points in control group. During the first 6 days after operation there were obvious differences in radiant heat stimulus between control group between the other groups (P < 0.05); During 14-22 days after operation, mechanical pain threshold and radiant heat threshold between p38β-SODN group and Model group were significantly changed compared with that in control group (P < 0.05). However, the differences were not remarkable between control group and p38β-ASODN group (P > 0.05). The expression of p38β protein in lumbar spinal cord was significantly higher between p38β-SODN group and Model group were significantly changed compared with that in control group (P < 0.05). However, the differences were not remarkable between control group and p38β-ASODN group (P > 0.05). The expression of p38β protein in lumbar spinal cord was significantly higher between p38β-SODN group and Model group than that in control group (P < 0.05). There was no significant difference in p38β protein expression between p38β-ASODN group and control group (P > 0.05). Conclusions: Hyperalgesia induced by bone cancer can be inhibited by intrathecal administration of p38β antisense oligonucleotide, which is achieved by reducing expression of p38β protein.

Keywords: Bone cancer pain, antisense oligonucleotides, p38beta, hyperalgesia, spinal dorsal horn

Introduction

Bone pain associated with cancer is a serious clinical health problem which is difficult to treat, and its mechanisms are not well understood [1, 2]. Circumstantial evidences suggest that progress has been made in understanding the changes of mitogen-activated protein kinase (MAPK) pathway in the induction and maintenance of chronic pain states [3-5]. In particular, p38 MAPK is typically activated by cellular stress and proinflammatory cytokines [6-9], and plays a critical role in inflammatory responses and spinal sensitization [10-12]. Four isoforms of p38 MAPK have been identified, each the product of distinct genes: p38α, 38β, p38γ and p38δ [5, 13, 14]. Among the p38 family members, p38α and p38β are two of the major isoforms in the mature nervous system. p38α is the most abundant isoform in the DRG and spinal cord [15]. We demonstrated that p38β was mainly expressed in spinal microglia after bone cancer pain was developed [16]. Inhibition of p38 pathways has been shown to effectively attenuate inflammatory and neuropathic pain in different animal models [15, 17-19]. Development of knockdown for p38 pathways to target neurons and glial cells may lead to new therapeutic strategy for pain management.

The antisense strategy is a better gene therapy technique and could be a useful tool for the study of endogenous gene regulation [20, 21].
Pathway analyses demonstrated that the phosphorylation of p38 and its downstream target CREB was inhibited by the antisense oligodeoxynucleotide (AS-ODN) [22] and knockdown of p38β with antisense oligonucleotides prevented acute pain sensitization [10]. However, few studies have focused on the role of p38β-ASODN in bone cancer pain.

We previously reported that p38β in the spinal cord participates in pain hypersensitivity using the bone cancer pain model [16]. In this study, we set out to investigate whether intrathecal p38β-ASODN attenuates experimental bone cancer pain and suppresses glial activation in the bone cancer pain model. The results indicate that the inhibitory effect of intrathecal p38β-ASODN on bone cancer pain is largely mediated by the downregulation of p38β.

Materials and methods

Animals

Adult female Sprague-Dawley rats weighing 200-250 g were housed at a constant ambient temperature of 24°C ± 1°C, humidity of 40%-70% under a 12 h light/dark cycle and given food and water ad libitum. The rats were individually housed in plastic cages with wood-chip bedding for at least 1 day before surgery. All experimental procedures were approved by the Institutional Animal Care and Use Committee, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology and were conducted in accordance with the NIH guide for the care and use of laboratory animals and the Ethical Issue of the IASP [23].

Cell preparation

MADB-106 mammary gland carcinoma cells were donated by Page GG and LY Liu and prepared as described previously [16, 24]. Cells were diluted in Hank’s medium to the required concentration for injection (3 × 10³ cells/10 μl) and kept on ice.

38β antisense oligodeoxynucleotides

Antisense and missense oligonucleotides to the rat p38β subunit were designed according to the cloned 5’ end fragment of the rat p38β gene [10]. The 38β sense ODN sequence was 5’-TCCACGCGAGGAGGACATAC-3’ and the antisense ODN sequence was 5’-GTATGTCCTCCTCGCGTGA-3’. 2’-O-Methoxyethylribose (MOE)-modified phosphodiester/phosphorthioate (PO/PS) chimeric oligonucleotides and MOE-modified phosphorothioate (PS) oligonucleotides crossreactive with rat 38β were synthesized, purified, and provided by Shanghai Bioengineering Technology Co. [22, 25, 26]. The wings of these chimeric ASO consist of 2’-MOE modified PO linkages whereas the gap consists of 2’H/PS nucleotides demonstrating RNase H-dependent antisense activity [22]. Synthetic oligonucleotides were dissolved in artificial cerebrospinal fluid (ACSF) to a final concentration of 20 µg per 10 µl before intrathecal administration. The ACSF contained (µM): Na⁺, 151.1; K⁺, 2.6; Mg²⁺, 0.9; Ca²⁺, 1.3; Cl⁻, 122.7; HCO₃⁻, 21.0; HPO₄²⁻, 2.5 and was bubbled with 95% O₂/5% CO₂ before use to adjust the final pH to 7.2.

Animal groups and surgical procedures

Rats were randomly divided into five groups (n = 10). Group Naive received neither intra-tibial operation nor intrathecal catheter implantation. Group Control received intra-tibial injection of 3 µL Hank’s solution; Group Model, group p38β-SODN and group p38β-ASODN received intra-tibial injection of 3 µL MADB-106 mammary gland carcinoma cells of rats (4.8 × 10⁹ cells µl). From the 14th day after injection, p38β-SODN and p38β-ASODN (20 µg per 10 µl) were administered intrathecally into group p38β-SODN and group p38β-ASODN on each of six consecutive days, respectively, while ACSF (10 µl) was injected into group Control and group Model by intrathecal catheter. In group Control and group Model, animals were allowed to survive for 22 days after surgery (n = 5 for immunohistochemistry and n = 5 for Western blotting).

All procedures were performed under pento-barbital anesthesia (50 mg/kg, i.p.). After 1 week after the implantation of the intrathecal catheter, rats were anesthetized and 3 µL solution was injected into the tibial bone of the left hind paw using 0.3 ml insulin syringe with 29.5 gauge needle, as described previously [16, 27].

The implantation technique of the intrathecal catheter was modified and performed [28, 29]. Briefly, after rats were adequately anesthetized, a PE10 catheter (o.d. = 0.61 mm) was inserted into the subarachnoid space between the L5 and L6 vertebrae, with its tip at the lumbar enlargement. The proper location of the
Figure 1. Effects of p38β-ASODN treatment on mechanical hyperalgesia. In first 12 days after intra-tibial operation, PWT in Model group was not significantly different from that in Control group ($P > 0.05$), whereas on 14-22 day after operation, differences were remarkable between the two groups ($^*P < 0.01$). On 14-22 day after operation, PWT in p38β-SODN group was not significantly different from that in Control group ($P > 0.05$), whereas significant differences in PWT were found between p38β-ASODN group and p38β-SODN group ($^*P < 0.05$), and there were obvious differences in PWT between p38β-ASODN group and Model group ($^*P < 0.05$). Data are expressed as mean ± SEM.

Figure 2. Effects of p38β-ASODN treatment on thermal hyperalgesia. No significant differences in radiant heat threshold were found at all-time points in control group. During the first 6 days after operation there were obvious differences in radiant heat stimulus between control group between the other groups ($^*P < 0.05$); During 14-22 days after operation, radiant heat threshold between p38β-SODN group and Model group were significantly changed compared with that in the p38β-ASODN group ($^*P < 0.05$). Data are expressed as mean ± SEM.
Figure 3. p38β protein expression of the lumbar spinal cord on day 22 after operation. No significant differences in p38β protein expression were found between p38β-ASODN group and Control group, between Model group and p38β-SODN group, respectively. p38β protein in Model group evidently augmented when compared with that in Control group (*P < 0.01), whereas p38β protein in p38β-ASODN group significantly decreased when compared with that in Model group (*P < 0.01). p38β protein in p38β-ASODN group was not significantly different from that in Control group at day 22 after operation (P > 0.05).

Western blotting

On 22 days after the intra-tibial injection, the lumbar spinal cord (L4-6) was removed. Total proteins from lumbar spinal segment were prepared by addition of 1 ml of ice cold solubilization buffer containing protease inhibitors. The tissue was homogenized. After being placed on ice for 30 min, the homogenate was centrifuged at 12,000 × g for 30 minutes at 4°C. The supernatant was assayed for protein content using the BCA assay method and stored at -20°C. Total protein (60 μg) was electrophoresed on a 6% sodium dodecyl sulfate polyacrylamide gel, as suggested by the manufacturer. After electrophoresis, the proteins were transferred and blocked with 5% nonfat dry milk. The primary antibody (rabbit anti-p38β, 1:500) was added and incubated for 2 h at room temperature in fresh blocking buffer. The membrane was washed for 30 minutes in washing buffer at room temperature, before the secondary antibody (1:500 dilution of alkaline phosphatase (AKP) coupled goat anti-rabbit immunoglobulin G (KPL, USA) was added for 1 h at room temperature in blocking buffer. The membrane was washed in washing buffer for another 30 min and the antibodies were then revealed using western blot reagent plus. For analysis, the blots were scanned and quantified with software and the results were expressed as the ratio of p38β immunoreactivity to β-actin immunoreactivity [37].
**Data analysis**

Investigators were blinded to all treatments in all tests. All values were expressed as the mean ± standard error of the mean (S.E.M.) and subjected to statistic evaluation using one-way analysis of variance (one-way ANOVA) followed by post hoc comparison (Student-Newman-Keuls test) to confirm significant differences between the groups. *P* < 0.05 is set as the level of statistical significance.

**Results**

**Physiological functions**

All rats kept on good health after surgery as assessed by general activity, normal weight gain, and grooming. Rats treated with p38β-ASODN consumed similar amount of food and fluid compared with p38β-SODN or ACSF over the entire observation period (*P* > 0.05). No significant difference was observed in terms of body weight, rectal temperature, and respiratory rate among four groups (*P* > 0.05).

**p38β-ASODN reverses mechanical and thermal hyperalgesia**

Model group developed a marked hypersensitivity to innocuous due to mechanical and thermal stimulation of the lateral surface of the left hind paw (sural nerve skin area) on 14 d after surgery (Figure 1). During the first 6 days after operation there were obvious differences in radiant heat stimulus between control group between the other groups (*P* < 0.05, Figure 2); During 14-22 days after operation, mechanical pain threshold and radiant heat threshold between p38β-SODN group and Model group were significantly changed compared with that in control group (*P* < 0.05). However, the differences to mechanical and thermal stimulation were not remarkable between control group and p38β-ASODN group (*P* > 0.05). Comparison among the four groups, the mechanical withdrawal threshold in p38β-ASODN group was significantly higher than that in Model group or p38β-SODN group on 22 days after surgery (*P* < 0.01, Figures 1 and 2).

**p38β-ASODN decreased bone cancer pain-induced p38β expression**

Effect of p38β-ASODN on spinal p38β protein expression was evaluated by western blotting (n = 5 each group, Figure 3). No significant differences in p38β protein expression were found at 22 d after operation between Control group and p38β-ASODN group, between Model group and p38β-SODN group, respectively. At day 22 after operation, p38β protein in Model group evidently augmented when compared with that in Control group (*P < 0.01), whereas p38β protein in p38β-ASODN group significantly decreased when compared with that in Model group (*P < 0.01). p38β protein in p38β-SODN group was not significantly different from that in Control group at 22 d after operation (*P > 0.05).

**Intrathecal p38β-ASODN suppressed glial activation in the bone cancer pain model**

A low-power image showed that the activation of GFAP and OX-42 was observed among four groups at 22 d after operation (n = 5 each group, Figure 4). And activated astrocytes demonstrated profoundly cell proliferation and hypertrophy in Model group, whereas the resting astrocytes displayed small round nuclei and slender processes in Control group. The result found that p38β-ASODN treatment markedly inhibited the activation of GFAP and OX-42 at 22 d after operation. In terms of immunohistochemical scores (Figure 4), there was no significant difference for GFAP and OX-42 immunostaining in spinal dorsal horns between Model group and p38β-SODN group, where there was significant difference for GFAP and OX-42 immunostaining between Model group and p38β-ASODN group.

**Discussion**

Our data demonstrated that (1) rat with down-regulation of spinal p38β are viable with no obvious health problems; (2) intrathecal injection of p38β-ASODN decreased mechanical and thermal hyperalgesia in bone cancer pain animals; (3) bone cancer pain significantly increased the expression of p38β protein in the spinal cord; (4) expression of p38β protein in the spinal cord was significantly down-regulated by administration of p38β-ASODN; (5) intrathecal injection of p38β-ASODN significantly reduced the activation of spinal glia. Therefore, these results suggest that p38β-ASODN may alleviate mechanical allodynia and thermal hyperalgesia through the p38β-related pathway. Beardmore et al reported that mice with knockouts of spinal p38β were viable with no
obvious health problems, suggesting that this mild phenotype can be compensation between different p38 isoforms [38]. Thus, down-regulation of p38β inhibits spinal nociceptive processing and represents a potential target for bone cancer pain therapy.

In agreement with our previous findings [16] and earlier reports by Dickenson and colleagues [39], the animals injected with MADB-106 mammary gland carcinoma cells into the tibia of rats showed progressive development of evoked mechanical and thermal hyperalgesia on the ipsilateral hindpaw. The contralateral hindpaw responses were the same as the sham group responses on both hindpaws and remained stable throughout the postoperative period, indicating that none of the behavioral tests cause tissue damage or hypersensitivity. These results were identical with previous published findings [40-42].

Fitzsimmons et al reported that peripheral inflammation-evoked hyperalgesia is prevented by downregulation of p38β by using intrathecal antisense oligonucleotides, suggesting that activation of spinal p38β may involve acute facilitatory processing [43]. Svensson et al showed that down-regulation of spinal p38β prevented nocifensive flinching evoked by intraplantar formalin injection and hyperalgesia induced by intrathecal injection of substance P [10]. We previously demonstrated that the p38 isoforms were distinctly expressed in dorsal horn of spinal cord: p38β in microglia and p38alpha in neurons [16]. Results of the current study demonstrated that mechanical and thermal hyperalgesia induced by bone cancer were inhibited by intrathecal administration of p38β antisense oligonucleotide, which is achieved by reducing expression of p38β protein and suppressing glial activation.
In conclusion, our data demonstrate the specific role of spinal p38β in mediating bone cancer pain. Thus, we conclude that therapeutic intervention targeting p38β may provide a novel approach to suppress spinal glial activation associated with bone cancer pain.

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Disclosure of conflict of interest

None.

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References


p38β antisense oligonucleotide and bone cancer

