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Osteopontin expression and function within the dorsal root ganglion

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Abstract

Osteopontin expression has previously been demonstrated in the adult rat dorsal root ganglion, although its function remains unclear. Here, we demonstrate, using real-time reverse transcription-polymerase (RT-PCR) chain reaction, that osteopontin mRNA expression is increased 1 and 3 weeks following sciatic nerve section (axotomy). Further, immunohistochemical staining suggests that this increase is restricted to neurons already expressing the protein. Osteopontin knock-out animals have significantly increased mechanosensory thresholds in the intact adult compared with the wild-type controls; however no differences in allodynia are noted between genotypes using a model of neuropathic pain. Lastly, exogenous recombinant osteopontin has no effect on neurite outgrowth from adult wild-type sensory neurons, nor were differences in neurite outgrowth observed in osteopontin knock-out animals compared with wild-type controls.

Keywords

dorsal root ganglia; nerve injury; neuropathic pain; neurite outgrowth; osteopontin

Introduction

Osteopontin is a sialoprotein first identified in the bone in 1986, with an approximate molecular mass of 32.5 kDa and an Arg-Gly-Asp (RGD) cell-binding sequence [1]. Osteopontin has been shown to bind a number of integrins, which include $\alpha_4\beta_1$ [2], $\alpha_8\beta_1$ [3], $\alpha_9\beta_1$ [4], $\alpha_v\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ [5], in both an RGD and a non RGD-dependent manner [6,4]. These integrins perform different functions, including roles in cell attachment and migration, chemotaxis and intracellular signalling [3,5], and the modulation of neuronal regeneration following injury [7,8]. Osteopontin also binds to splice variants of the hyaluronan receptor CD44 and may then initiate chemotaxis in a dose-dependent, RGD-independent manner [9]. CD44 has been implicated in numerous cellular functions including wound healing, embryogenesis, extracellular matrix adhesion and leukocyte activation (for a review see [10]).

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Conflict of interest: None.

Osteopontin has a widespread distribution in the body, including the central and peripheral nervous systems. Both the adult rat dorsal root ganglion (DRG) and trigeminal ganglion contain abundant osteopontin immunoreactive neuronal cell bodies and axons; a quarter of DRG neurons were immunoreactive for osteopontin [11]. Many of these large diameter neurons colocalize with the parvalbumin, but not with calcitonin gene-related peptide. Axotomy induces a seven-fold increase in osteopontin expression in the superior cervical ganglion [12], but to date no such increase has been described in the DRG.

This study used a variety of molecular, cellular and behavioural techniques to further elucidate the localization and the function of osteopontin in the sensory neurons of the adult mouse DRG.

Methods

Osteopontin knock-out mice

Breeding colonies of osteopontin knock-out and wild-type mice on the B6 × 129SvJ strain were established and maintained at the University of Bristol. Details of the strain and breeding history have previously been published [13]. In brief, a pMC1-Neo cassette in reverse orientation was inserted into the *EagI* site in osteopontin exon 6 and, following germ line transmission of the mutation, was bred to and has been maintained at homozygosity. Mice were genotyped using the following primers: osteopontin sense: 5'-AACAAGAGGCCCGTTTCATTAGCT-3'; osteopontin antisense: 5'-AAGCTATCACCTCGGCCGTTGG-3'; neomycin: 5'-CCGCTTTTCTGGATTCATCGACTGT-3'.

Age, sex, and strain-matched adult mice (10-12 weeks old, 25-30 g) were used in all experiments.

Surgery

Animals were fed standard chow and water *ad libitum*. All the experiments were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986.

Mice were anaesthetised with Hypnorm (0.315 mg/ml fentanyl citrate + 10 mg/ml fluanisone; Janssen, Beerse, Belgium)/Hypnovel (5 mg/ml midazolam; Roche Applied Sciences, Burgess Hill, West Sussex, UK)/water at a ratio of 1:1:2 at 4 µl/g.

As described previously [14], a modification of the spared nerve injury (SNI) model of neuropathic pain was used. An incision into the lateral right hind leg, just above the level of the knee, exposed the three terminal branches of the sciatic nerve: the common peroneal, tibial and sural nerves. The common peroneal and sural nerves were ligated tightly with 7/0 silk and approximately 5 mm of nerve stump distal to the ligation was removed to prevent regeneration occurring. The tibial branch remained untouched during the procedure. The overlying muscle and skin was sutured, and the animals were allowed to recover.

Right side axotomy was performed on wild-type mice (10-12 weeks old). Under anaesthetic (see above) the sciatic nerve was exposed and sectioned at the mid-thigh level. Approximately 5 mm of the distal nerve stump was removed before the overlying muscle and the skin was sutured and the animal was allowed to recover.

Real-time reverse transcription-polymerase chain reaction

Total RNA from 12 mice/group was isolated from control and axotomized L4 and L5 wild-type DRG using an RNeasy kit (Qiagen, Crawley, West Sussex, UK), and cDNA was generated using random hexamers (Applied Biosystems Foster City, California, USA). Real-

time quantitative SYBR-green reverse transcription-polymerase chain reaction (RT-PCR) assays [15] used primer sets designed using the default parameters of the Primer Express software (Applied Biosystems). Osteopontin sense 5'-GAGTTTCCAGGTTTCTGATGAACA-3' and osteopontin antisense 5'-TTAGACTCACCGCTCTTCATGTG-3' primers correspond, respectively, to nucleotides 268-591 and 648-626 of BC057858 [16]. Endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA primers were 5'-GCAGTGGCAAAGTGGAGATTG-3' and 5'-CTGGAACATGTAGACCA-TGTAGTTGA-3' that correspond, respectively, to nucleotides 111-131 and 184-159 of M32599 [17]. The reactions were performed on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems) and used standard cycling conditions (95°C for 10 min followed by 50 cycles of 95°C for 15 s and 60°C for 60 s).

Behavioural testing

In all the tests, the examiner was blind to the genotype of the mice.

Thermal thresholds were measured according to the method of Hargreaves *et al.* [18]. Animals were habituated to the environment, a transparent Perspex holding box (Ugo Basile, Varese, Italy), for at least 1 h. Testing was performed on three separate occasions before surgery. The hind paws were exposed to a beam of radiant heat through the box, and the latency of withdrawal was recorded automatically. Each hind paw was measured 5 times with an interval of at least 5 min between measurements.

A series of von Frey filaments (Stoelting, Wood Dale, Illinois, USA), calibrated to produce from 0.005g to a maximum of 3.63g of pressure when applied were used to measure mechanical thresholds. Animals were placed into Perspex enclosures with an elevated mesh floor (Ugo Basile) and habituated for at least 1 h before testing.

Mechanical sensitivity was determined using the up/down testing paradigm on each hindpaw. Briefly, the filaments were applied to the plantar surface of the animal's hindpaw in the ascending order of pressure. Once a response (defined as any lifting, shaking or biting of the stimulated paw) was observed, filaments of lower pressure were applied until a response was no longer noted. Filaments of increasing pressure were then applied, and the cycle was repeated. In this way, the threshold force required to elicit a withdrawal response to 50% of stimulations was determined [19,20].

Immunohistochemistry

One week after axotomy, the animals were intracardially perfused with 4% paraformaldehyde/phosphate-buffered saline (PBS) and the spinal columns were removed and postfixed for 4 h. Ipsilateral and contralateral L4 and L5 DRG were dissected and equilibrated in 20% sucrose overnight at 4°C, embedded in optimal cutting temperature (OCT) mounting medium, frozen on dry ice and sectioned at 16 µm with a cryostat.

Sections were blocked and permeabilized in 10% normalized donkey serum/PBS/0.2% Triton X-100 (PBST) for 1 h at room temperature. They were then incubated in goat polyclonal antibody to osteopontin (R&D Systems, Abingdon, Oxfordshire, UK) at 1:800 in PBST overnight in a humid chamber, washed 3 × 10 min and incubated in either donkey anti-goat fluorescein isothiocyanate (FITC) (Jackson, Immunoresearch, Newmarket, Suffolk, UK) at 1:200 or anti-goat Cy3 (Jackson) at 1:500 for 3 h at room temperature.

When appropriate, sections were also incubated with antibodies raised in rabbit against CGRP (Affiniti, Biomol, Exeter Devon, UK) or neurofilament (Chemicon, Millipore, Chandlers Ford, Hampshire, UK), followed by incubation with either donkey anti-goat FITC (Jackson) at 1:200 or anti-goat Cy3 (Jackson) at 1:500 for 3 h at room temperature, or with

biotin-conjugated isolectin-B4 (IB4) at 10 µg/ml (Sigma-Aldrich, Gillingham, Dorset, UK) in PBST, followed by extravidin FITC at 1:100 (Sigma).

After washing, the sections were mounted in Vectashield (Vector Laboratories, Peterborough, Cambridgeshire, UK). Images were taken with a Leica fluorescent microscope (Leica Microsystems, Milton Keynes, Buckinghamshire, UK) and analyzed in Adobe Photoshop.

Neurite outgrowth

Animals were killed by cervical dislocation. DRG from the lumbar, thoracic and cervical regions were removed aseptically and collected in Dulbecco's modified Eagle's medium (DMEM)/F12 medium. As described previously [21], ganglia were treated with 0.25% collagenase P for 1 h at 37°C, washed in PBS and subjected to trypsin/ethylenediaminetetraacetic acid for 10 min at 37°C. After washing in the medium containing trypsin inhibitor, ganglia were mechanically dissociated by trituration. Cells were then centrifuged and resuspended in DMEM/F12 medium, supplemented with 5% horse serum, 1 mM glutamine and 10 ng/ml gentamycin. To remove the glial cells and eliminate debris, cells were plated onto six-well plates previously treated with 0.5 mg/ml polyornithine and incubated overnight at 37°C. The medium was removed and discarded. The neurons were removed from the surface with a jet of fresh medium. Following centrifugation, cells were plated on 24-well plates previously treated with 0.5 mg/ml polyornithine and 5 µg/ml laminin and maintained for 8 h at 37°C in a humidified incubator.

Cells were cultured in DMEM/F12 supplemented medium described above, with or without the addition of 50, 100 or 300 nM osteopontin (R&D Systems) for 4 or 8 h.

At the end of the experiment, the medium was removed and the cultures were washed with PBS before fixation with 4% paraformaldehyde/PBS for 20 min at room temperature. Cells were visualized by phase-contrast microscopy. The percentage of cells bearing neurites and neurite length was estimated using NIH Image (Scion, Frederick, Maryland, USA).

Statistics

Data are presented as the mean±SEM. Student's *t*-test was used to analyse the difference in baseline thermal withdrawal thresholds. Analysis of variances and nonparametric Mann-Whitney *U* post-hoc tests were used as appropriate to analyse differences between the genotypes.

Results

Real-time reverse transcription-polymerase chain reaction

Semiquantitative real-time RT-PCR reactions for osteopontin mRNA were performed 3 days, 1 week and 3 weeks after axotomy on adult mouse DRG. Results demonstrated no change in mRNA levels after 3 days. In contrast, a 36% increase was detected at both 1 and 3-weeks after axotomy (*n*=2).

Immunohistochemistry

Immunohistochemistry demonstrated that 13% of the adult mouse DRG neurons express the osteopontin protein. High levels of colocalisation between osteopontin and neurofilament (NF200) were observed (Fig. 1) whereas colocalisation was not detected between osteopontin and either CGRP or IB4 (data not shown).

No significant differences in the number of osteopontin immunoreactive cells were observed in ipsilateral DRG compared with contralateral controls at either 3 days or 1 week following axotomy (data not shown).

Nociceptive testing

Mechanical and thermal withdrawal thresholds were tested in intact adult wild-type and osteopontin knock-out animals. A significant increase in mechanical withdrawal threshold to 1.347 ± 0.13 g was observed in the osteopontin knock-out mice compared with the wild-type controls 1.024 ± 0.09 g ($P < 0.005$). In contrast, no differences in thermal latencies were observed between the two genotypes (wild type mean: 8.34 ± 0.56 s, osteopontin knock-out mean: 7.63 ± 0.59 s, $P > 0.05$).

Mice were then tested for the development of mechanical allodynia for up to 2 weeks after the SNI. One day after SNI, all mice had developed marked and significant mechanical allodynia ipsilateral to the injury on the lateral surface of the hindpaw. No significant differences in the degree, or time course, of the allodynia were noted between the genotypes (Fig. 2).

Neurite outgrowth

To determine whether osteopontin is involved in neurite outgrowth from adult sensory neurons, we tested its ability to support neuritogenesis at a variety of different concentrations and time points. No significant effects of 50 nM osteopontin were noted in wild-type (control: 137.4 ± 2.63 μ m vs. 50 nM osteopontin: 131.6 ± 2.28 μ m; $P > 0.05$, $n=3$) or osteopontin knock-out (control: 139.7 ± 2.82 μ m vs. 50 nM osteopontin: 133.9 ± 2.62 μ m; $P > 0.05$, $n=3$) cultures, 8 h after replating on laminin. The addition of osteopontin also did not produce a significant change in the number of cells with neurites in the wild-type (control: $23.4 \pm 1.04\%$ vs. 50 nM osteopontin: $23.1 \pm 0.9\%$; $P > 0.05$, $n=3$) or osteopontin knock-out (control: $23.5 \pm 1.10\%$ vs. 50 nM osteopontin: $21.9 \pm 0.96\%$; $P > 0.05$, $n=3$) cultures. Similarly, neither 100 nor 300 nM osteopontin affected neuritogenesis in wild-type or osteopontin knock-out cultures at 4 or 8 h after replating (data not shown).

Discussion

This paper is the first description of osteopontin expression in the adult mouse DRG and is similar to that reported previously in the rat. Osteopontin colocalizes with neurofilament protein, which is a marker of mechanosensory neurons. This study found no colocalization with cells expressing CGRP, which is expressed by small diameter sensory neurons, nor IB4 (a marker for nonpeptidergic unmyelinated afferents). Ichikawa *et al.* [11] demonstrated a similar lack of osteopontin and CGRP colocalization in the rat.

Here, we demonstrate that, following sciatic nerve axotomy, osteopontin mRNA expression in the DRG is increased ~35%, and the increase is maintained over at least a 3-week period. Other studies have shown upregulation in expression within the rat superior cervical ganglion, [11,12] but not the DRG. Despite the increase in mRNA levels, the number of osteopontin immunoreactive neurons did not change. Possible reasons for this include: (a) any increase in protein expression is confined to osteopontin expressing neurons or (b) the increase at the protein level is below the level of detection by the immunoassay.

The finding that osteopontin is expressed in a subset of large diameter DRG, neurons that are likely to be mechanoreceptors, led us to investigate the role played by osteopontin in nociception. Mechanical thresholds in intact adult animals were increased in the osteopontin knock-out compared with wild-type controls, although no such deficits were noted in thermal thresholds. Further, no differences in mechanical allodynia were noted between the

genotypes after an SPN model of neuropathic pain. Taken together, these data imply that osteopontin may either play an, as yet unknown, role in mechanical sensitivity in the intact (uninjured) adult animal or possibly may modulate the survival of a subset of mechanosensors at some point during development of the DRG. Further studies are therefore warranted on osteopontin expression in the DRG at different points during development, and to quantify in greater detail any possible differences in the number of mechanosensory neurons in osteopontin knock-out animals.

Osteopontin interacts with several integrins [22], many of which are closely linked to neurite outgrowth. The $\alpha_4\beta_1$ -integrin heterodimer increases the neurite extension in peripheral and central ganglia [7], and $\alpha_v\beta_3$ has also been shown to be involved in neurite outgrowth [23]. Recombinant, unmodified osteopontin has previously been shown to increase neurite outgrowth in the trigeminal ganglia [7] and to decrease outgrowth in rat DRG explants [7,24]. This study, however, found no change in neurite outgrowth in dissociated cultures from adult wild-type or osteopontin knock-out mice at chosen time points following the addition of a range of osteopontin concentrations. We conclude that osteopontin either does not play a role in neuritogenesis from adult sensory neurons and/or that there is a degree of genetic redundancy between osteopontin and other molecules that are capable of replacing its function in the osteopontin knock-out animal.

Conclusion

This study has demonstrated the upregulation of osteopontin mRNA in the DRG following injury, although no conclusive function for this increased expression has been demonstrated. Our data, unexpectedly, imply that osteopontin may play a role in mechano-sensation in the uninjured adult mouse. The possibility that osteopontin may play a developmental survival role in a subset of DRG neurons is compatible with its previously defined antiapoptotic role in the central nervous system [22,25].

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Fig. 1. Immunohistochemical localization of neurofilament NF200 (a) and osteopontin (b) in the dorsal root ganglion, demonstrating high levels of colocalization between the two proteins (examples marked by arrows).

Fig. 2.

Time course of mechanical allodynia-like behaviour in wild-type and osteopontin knock-out animals after spared nerve injury (SNI). Presurgery 50% withdrawal thresholds are denoted as day 0. Prespared nerve injury thresholds are significantly different between genotypes ($P < 0.005$). Following spared nerve injury, wild type (WT) and osteopontin knock-out (KO) animals develop robust allodynia (Mann–Whitney U $P < 0.001$ at day 1). *Osteopontin knock-out baseline compared with wild-type baseline. No significant differences in withdrawal thresholds were noted between the two genotypes after surgery.