Article highlights.

- Periodontal tissue regeneration aims to reconstruct the ligament structures in the tooth-supporting connective tissue.
- Periodontal ligament (PDL) stem cells can be used to reconstitute the PDL structure, including extracellular components.
- PDL stem cells (PDLSCs) express mesenchymal stem cell markers STRO-1 and CD146/MUC18 and show a similar phenotype to dental follicle stem cells (DFSCs).
- PDL cell sheets may induce periodontal regeneration, including reforming the PDL and cementum, and could provide an *in vivo* treatment for periodontal disease.
- The local application of human recombinant cytokines such as fibroblast growth factor (FGF)-2, platelet-derived growth factor (PDGF), bone morphogenetic protein (BMP)-2 and TGF-β stimulates and promotes the regeneration of periodontal tissues in animal models.
- Fibrillin-1 microfibril network is also important for PDL function and maintaining connective tissue integrity. Targeting the extracellular matrix (ECM) and fibrillin-1 microfibrils may offer a new administration therapy for periodontal disease.

This box summarizes key points contained in the article

over 60 years of age [4]. Furthermore, this disorder is the major cause of tooth loss in adults of over 40 years and its more severe forms has a worldwide prevalence of up to 20% according to the World Health Organization [5]. Blocking the progression of periodontal disease has been achieved by mechanically removing bacterial biofilm with conventional periodontal and/or surgical treatments. These treatments can reduce the destruction of periodontal tissue and diminish inflammation in the affected region. However, achieving adequate periodontal tissue regeneration remains a problem, particularly in cases where the disease has caused large defects in the periodontal tissue.

The current advances in future regenerative therapies have been influenced by many previous studies of embryonic development, stem cell biology and tissue engineering technologies [6-9]. To restore the partial loss of organ functions and to repair damaged tissues, attractive concepts that have emerged in regenerative therapy is stem cell transplantation into various tissues and organs [10] and cytokine therapy, which has the potential toinduce the activation and differentiation of tissue stem/progenitor cells [11]. Tooth tissue stem cells and the cytokine network that regulates tooth development, and dental tissue cell growth and differentiation, have been well characterized at the molecular level [12,13]. The regeneration of periodontal tissues is being made clinically possible by the transplantation of mesenchymal stem cells which can differentiate into PDL cells, cementoblasts and osteoblasts, or through the local application of cytokines to stimulate the proliferation and differentiation of these stem cells [14-17]. Although these therapies are effective and contribute to periodontal tissue repair, these interventions will likely be improved by an enhanced understanding of the development of periodontal tissues, particularly those involved in the formation of PDL, cementum and alveolar bone.

The ECM is a biologically active molecule composed of a complex mixture of macromolecules that, in addition to serving a structural function, profoundly affect the cellular physiology of an organism [18]. Previous findings have revealed that ECM components including type I collagen, type III collagen, lumican, decorin, periostin, f-spondin, tenascin-N and PLAP1/aspirin are highly expressed during PDL formation [19,20]. Since the ECM is regulated in a tissue-specific manner, these structures could enhance periodontal regeneration by promoting the differentiation of cells required for the synthesis of PDL, bone and cementum [21,22]. Among the ECM formations in the PDL, fibrillin-1, a major component of the microfibrils that regulate tissue integrity and elasticity, has been shown to contribute to the formation and maintenance of this ligament. An abnormal PDL structure in association with the progressive destruction of microfibrils has been observed in a Marfan's syndrome (MFS) mouse model and has characteristics that are similar to those of fibrillin-1 dysfunction [23]. These findings have strongly suggested that microfibril formation through fibrillin-1 assembly plays an important role in PDL formation and function. However, the molecular mechanisms of fibrillin-1 microfibril assembly remain unclear as the microfibril-associated molecule that regulates or stabilizes fibrillin-1 microfibril formation has not yet been identified. Recent findings have revealed that ADAMSL6β is essential for the development and regeneration of the PDL through the direct interaction of fibrillin-1 to promote microfibril assembly [23,24]. These findings have also suggested that the administration of fibrillin-1 microfibrils provides a novel therapeutic strategy for the treatment of periodontal disease.

We here review the present status of the periodontal tissue regeneration technologies that focus on the molecular mechanisms underlying development, regeneration and tissue engineering of periodontal tissue, and also discuss the potential of ECM administration therapy through the promotion of microfibril assembly as a novel therapeutic strategy for the essential functional recovery of periodontal tissue.

2. Development processes in periodontal tissue

The PDL has essential roles in tooth support, homeostasis and repair, and is involved in the regulation of periodontal cellular activities such as cell proliferation, apoptosis, the secretion of extracellular matrices, resorption and repair of the root cementum and remodeling of the alveolar bone [25-27]. To develop future methods to regenerate damaged PDLs, it will be important to understand the molecular basis of PDL development.

2.1 Molecular mechanisms underlying periodontal tissue development

The PDL is derived from the dental follicle (DF), which is located within the outer mesenchymal cells of the tooth germ and can generate a range of periodontal tissues including

the PDL, cementum and alveolar bone [21]. The DF is formed during the cap stage of tooth germ development by an ectomesenchymal progenitor cell population originating from the cranial neural crest cells [28]. Given the critical role that the progenitor cell population in the DF appears to play in the development of periodontal tissue, the developmental processes in this tissue are of considerable interest in terms of further understanding the biology of these cells (Figure 1). The differentiation of the DF proceeds as follows: i) during the tooth root-forming stage, the Hertwig's epithelial root sheath (HERS) comprising the inner- and outer-dental epithelia that initiate tooth root dentin formation is fragmented into the Malassez epithelium resting on the tooth root surface; ii) the DF migrates to the surface of the tooth root and differentiates into cementoblasts to form the cementum matrix [29,30]; iii) at almost the same time, the DF differentiates into the PDL on the cementoblasts in order to insert collagen fibers, known as Sharpey's fibers, into the cementum matrix. Fiber insertion also takes place along the alveolar bone and iv) both bone- and PDL-derived fibers finally coalesce in the PDL to form the intermediate plexus, which resembles tendinous tissue [31-33].

The DF has long been considered to be a source of multipotent stem cells (DFSCs), since these cells have the ability to migrate onto the tooth root surface to form periodontal tissue including cementum, PDL and alveolar bone during the tooth root-forming stage [32,34-37]. Previous studies have indicated that DF cells can form PDL-like tissues and cementum/bone-like structures after implantation into immunodeficient mice [38,39], supporting the notion that stem cells which can differentiate into PDL, cementoblast, osteoblast lineages are present in the DF [34,35]. To regenerate periodontal tissue, functional molecules which promote the differentiation of DFSCs into PDL need to be elucidated to enable a proper understanding of the mechanisms underlying periodontal tissue formation, including the pathways pertaining to PDL cell, cementum and alveolar bone differentiation.

2.2 Functional molecules involved in DF differentiation

Although the molecular mechanisms of DF development and differentiation remain to be determined, previous gene expression studies of mouse molar root development have suggested that some growth factors, including bone morphogenetic protein (BMP) 4, growth and differentiation factors (GDFs) 5, 6 and 7 [40-43], epidermal growth factors [44], *Shh* [45-47] and insulin-like growth factor (IGF)-1 [48], are involved in the growth or differentiation of the DF. Transcriptional factors such as *Scleraxis, Gli, Msx1, Msx2* and *Runx2* have also been shown to be involved in the differentiation of the DF into cementoblasts and in the mineralization of cementum [39,43,46,49]. Among these factors, GDFs and scleraxis are the most well characterized that are involved in tendon/ligament morphogenesis, suggesting that PDL development shares similar molecular mechanisms to those of tendon/ligament morphogenesis. With regard to cementogenesis/osteogenesis of the DF, treatment of this tissue with BMP-2 and BMP-7 has been found to induce mineralization ability. In addition, previous findings suggest that PDL cells harbor mineralization inhibitory mechanisms that enable them to maintain a ligament structure across the mineralized tissue, including the alveolar bone and cementum, during PDL development [50-52]. These observations strongly suggest that the tendon/ligament-related cytokines, the BMPs, and inhibitors of mineralization are linked to the restoration of the tendinous structure of the PDL. The mechanisms involving these factors may also have a role in preventing ankylosis of the PDL.

3. Regeneration therapies for PDL defects

A partial restoration of periodontal tissue has been achieved previously using a guided tissue regeneration (GTR) technique which provides an adequate space and favorable niche for the repair of periodontal defects using barrier membrane [53]. From the results of these GTR therapies, regeneration of the PDL has been shown to be critical for recovering the connection between the cementum on the root surface and the alveolar bone.

To regenerate periodontal tissue that has been destroyed by periodontal disease requires the recruitment of PDL stem cells (PDLSCs) to properly reconstitute the PDL structure including its extracellular components such as the collagen and elastic fibril systems [32,33]. Recent studies of stem/progenitor cells have provided considerable new insights that have furthered our understanding of PDLSCs, which can differentiate into periodontal tissue cell lineages such as PDL, cementum and alveolar bone [14,54]. PDLSCs will have utility for the future development of stem cell transplantation therapies and tissue engineering applications to restore periodontal organ function as they replace damaged areas with enriched and purified stem cells and thereby achieve PDL repair (Figure 2) [14]. The biological potential of PDLSCs to stimulate the regeneration of periodontal tissue can now be realized by the local application of human recombinant cytokines.

3.1 Stem cell therapies

PDLSCs have been isolated from human PDL tissue by single-colony selection and magnetic activated cell sorting. PDLSCs express the mesenchymal stem cell markers STRO-1 and CD146/MUC18, and can differentiate into cementoblast-like cells, adipocytes and fibroblasts [14]. In addition, PDLSCs show the capacity to generate a cementum/PDL-like structure and contribute to periodontal tissue repair on transplantation into immunocompromised rodents. Clonal PDLSC analysis has further revealed that these cells show a similar phenotype to DFSCs since they also express *RUNX-2, Col I, ALP, OPN, OCN, RANKL, OPG, scleraxis, periostin, Col XII* and *alpha-SMA* mRNAs [54]. Importantly, PDL tissue collected from one tooth can give rise to many stem cells because of their high proliferation capacity *ex vivo*. Recently also, it has been shown that the transplantation of autologous PDLSCs obtained from the

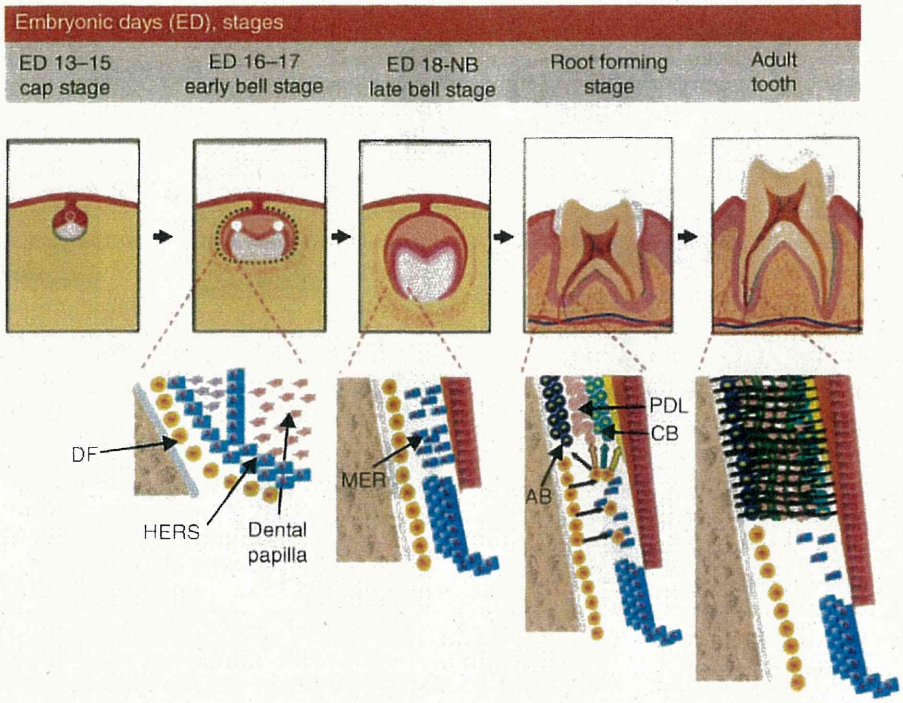

Figure 1. Developmental processes in the PDL (upper panel). PDL development originates from the DF located on the periphery of the tooth germ (arrows). The DF is generated from the dental mesenchyme during the cap stage of tooth germ development in the embryo. Development of the DF progresses during the early- and late-bell stages of the tooth germ, but no morphological changes are observed. Differentiation of the DF begins during the tooth root-forming stage and the mature PDL is subsequently formed (lower panel). DF differentiation commences after the tooth root dentin is formed by reciprocal interaction between the HERS and dental papilla. During tooth root dentin formation, the HERS is fragmented into the Malassez epithelial rest and the DF is then capable of migrating onto the root dentin to be differentiated into cementoblasts, PDL and alveolar bone to connect the tooth root and jaw bone (adult tooth).

PDL: Periodontal ligament; DF: Dental follicle; HERS: Hertwig's epithelial root sheath.

extracted teeth of miniature pigs can regenerate and repair a surgically created periodontal defect [55]. This finding suggests that PDLSCs obtained from an easily accessible tissue resource and expanded *ex vivo* using wisdom teeth might represent a feasible therapeutic approach to the reconstruction of tissues destroyed by periodontal disease.

In addition to the clinical application of stem cell transplantation, cell sheet engineering therapies for periodontal tissue regeneration are now being developed for clinical application [56,57]. In this technology, temperature-responsive dishes are used to harvest the cell sheets through a simple decrease in the temperature, thus avoiding the use of proteolytic enzymes [58]. The use of this method allows PDL cell sheets to be easily harvested and transplanted into periodontal defects *in vivo* [56,57,59,60]. PDL cell sheets have the potential to induce periodontal regeneration, including the reformation of the PDL and cementum. The available data also suggest that this technique has the appropriate efficacy for periodontal regeneration in patients with periodontal disease.

3.2 Cytokine therapies

Some new treatments that accelerate the regeneration of periodontal tissue by local application of human recombinant cytokines have now been established. This approach stimulates the proliferation and differentiation of stem cells/progenitors from the PDL into hard tissue-forming cells. The local application of human recombinant cytokines such as platelet-derived growth factor (PDGF) and IGF-1 [16,61], BMP-2 [62,63], TGF-β [64], osteogenic protein (OP)-1 [65] and brain-derived neurotrophic factor (BDNF) [66] stimulates and promotes the regeneration of regional periodontal tissue in animal models. The potency of PDGF-BB plus β-tricalcium phosphate (β-TCP, an osteoconductive scaffold) in periodontal tissue regeneration in human has also been recently reported [67]. In addition, a clinical Phase I study of fibroblast growth factor (FGF)-2 has shown that it stimulates the regeneration of periodontal tissue lost due to periodontal disease and demonstrated the safety of this treatment [15]. The results of this trial were clinically interpreted as a demonstration of

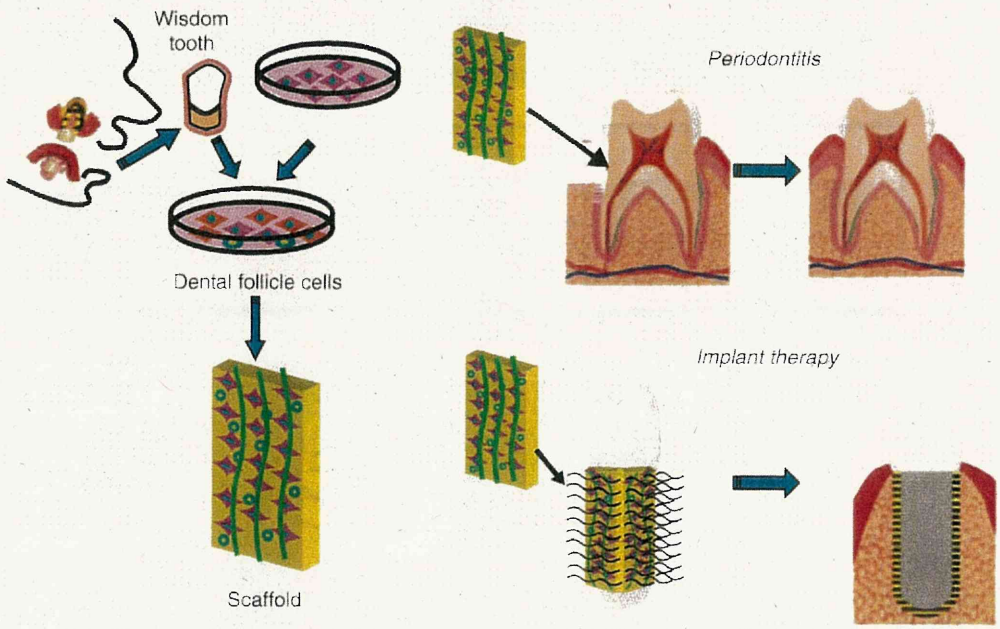

Figure 2. Potential approach to regeneration therapy for periodontal disease. By using cell sheet-engineering, stem cells obtained from the periodontal ligament (PDL) or dental follicle (DF) of wisdom tooth germ are harvested as temperature sensitive sheets for transplantation into periodontal tissue damaged by periodontal disease (upper panel). Stem cell sheets are also applied to dental implants accompanied by a bioengineered PDL that can recover the loss of periodontal tissue including the PDL, cementum and alveolar bone (lower panel).

the efficacy of FGF-2 in stimulating the regeneration of periodontal tissue. These findings collectively suggest that cytokine therapy has great clinical potential for achieving the partial regeneration of periodontal tissue.

4. Novel approaches to periodontal tissue regeneration using ECM administration therapy

ECM components organized in the PDL not only reflect the functional requirements of this matrix such as mechanical stress and storage of signaling molecules, but also regulate the tissue framework during development and regeneration [21]. Diseases affecting ECM function such as MFS have been shown to increase the susceptibility to severe periodontal disease due to a dysfunction of the PDL through a microfibril insufficiency, suggesting that fibrillin-1 microfibril formation plays a central role in PDL formation [68-74]. In addition, a new therapeutic concept has proposed that a fibrillin-1 microfibril insufficiency can be corrected by the administration of ECM components [23].

4.1 Periodontal disease and MFS

MFS is a severe, systemic disorder of connective tissue formation and can lead to aortic aneurysms, ocular lens dislocation, emphysema, bone overgrowth and severe periodontal disease [68,75,76]. MFS has an estimated prevalence of 1 in 5000 – 10,000 individuals [77]. Fibrillin-1 comprises one of the major insoluble ECM components in connective tissue microfibrils which provides limited elasticity to tissues and stores cytokines such as TGF-β [78,79] (**Figure 3A**). Various mouse models of MFS have now been established via gene targeting or missense mutations in which germline mutations in *fibrillin-1* lead to progressive connective tissue destruction due to fibrillin-1 fragmentation in association with an insufficiency of fibrillin-1 microfibril formation [72,74,75]. Hence, it is largely accepted that MFS is caused by insufficient fibrillin-1 microfibril formation in various connective tissues [76]. The study of PDL provides a useful experimental model not only for investigating the molecular pathogenesis of MFS, but also for evaluating novel therapeutic strategies for the improvement of microfibril disorders. This is because the principal elastic fiber system of the PDL, the oxytalan fiber, is composed of fibrillin-1 microfibrils and does not contain significant amounts of elastin [80-82]. Indeed, an abnormal PDL in association with progressive destruction of microfibrils is an obvious phenotype in the MFS mouse model [23]. Hence, PDLs will likely be more susceptible to breakdown in MFS compared with other elastic tissues composed of both elastin and fibrillin-1 (**Figure 3B**).

A structural insufficiency of fibrillin-1 microfibrils arises in MFS and leads to activation of TGF-β and its regulatory targets

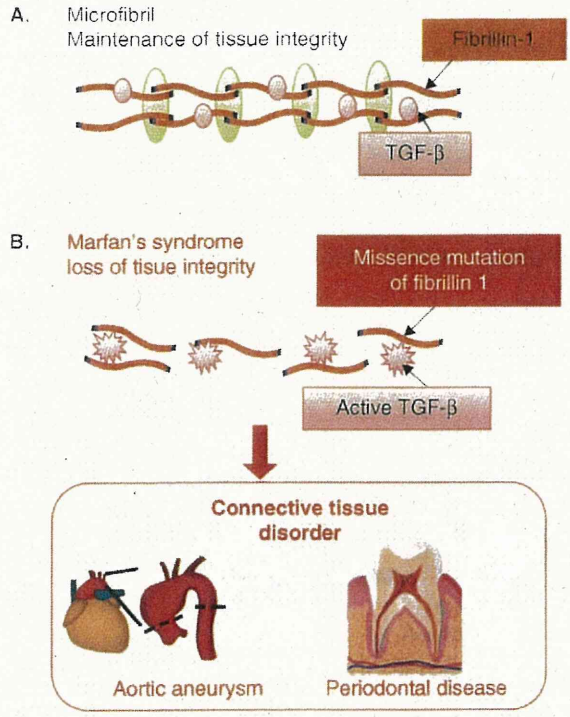

Figure 3. Schematic representation of the pathogenic mechanisms of MFS. A. Fibrillin-1 comprises insoluble extracellular matrix components in connective tissue microfibrils and provides limited elasticity to tissues through fibrillin-1 microfibril formation. **B.** Missense mutations in the *fibrillin-1* gene lead to fibrillin-1 fragmentation in association with an insufficiency of fibrillin-1 microfibril formation and the pathogenic activation of TGF-β. These abnormalities cause progressive connective tissue destruction including aortic root aneurysms that are life-threatening and severe periodontal disease.

(Figure 3B) [73]. Recently, deregulation of TGF-β activation has been shown to contribute to pathogenesis and systemic antagonism of TGF-β signaling has been observed to have a beneficial effect on MFS symptoms including alveolar septation and muscle hypoplasia [72]. These observations have indicated that the microfibril network plays an important role in not only PDL function but also in the recovery of periodontal tissue integrity and prevention of the pathogenic activation of TGF-β caused by the fibrillin insufficiency that arises in MFS. However, molecular mechanisms governing fibrillin-1 assembly during organogenesis have been hampered because of unanswered issue of the actual factor that drives microfibril assembly.

4.2 Administration of ADAMTSL6β serves as a microfibril therapy for repair of the PDL in an MFS mouse model

A disintegrin-like metalloprotease domain with thrombospondin type I motifs (ADAMTS)-like, ADAMTSL, is a subgroup of the ADAMTS superfamily and its members share particular protein domains with the ADAMTS protease, including thrombospondin type I repeats, a cysteine-rich domain and an ADAMTS spacer, but lack the catalytic and disintegrin-like domains. Among the novel ADAMTSL family molecules, ADAMSL6β is essential for the development and regeneration of the PDL [23]. ADAMTSL6β was recently found to associate with fibrillin-1 microfibrils through its direct interaction with the N-terminal region of fibrillin-1, and thereby promote fibrillin-1 matrix assembly both *in vitro* and *in vivo* [24]. Another study has indicated that fibronectin is an essential component during the assembly of fibrillin-1 through its interaction with the C-terminal region of fibrillin-1, thus suggesting the potential for improved microfibril assembly through the regulation of fibrillin-1-associated proteins including ADAMTSL6β [83,84]. In an animal model of MFS microfibril disorder [85], ADAMSL6β expression can rescue fibrillin-1 microfibril formation through the promotion of fibrillin-1 microfibril assembly (Figure 4A). More importantly, the local administration of ADAMTSL6β was found to be highly effective in accelerating the wound healing of periodontal tissues through the restoration of microfibrils (Figure 4B). Further evidence for the impact of ADAMTSL6β on microfibril assembly is its suppression of TGF-β signaling, a pathway which is known to contribute to elastolysis in MFS.

These findings have demonstrated that microfibril assembly induced by ADAMTSL6β is essential not only for fibrillin-1 microfibril restoration but also for the inhibition of the pathological activation of TGF-β. Thus, ECM administration therapy such as microfibril assembly could form the basis of a novel therapeutic approach to PDL regeneration and the treatment of periodontal disease in MFS patients.

5. Conclusions

Regenerative therapies for periodontal disease that use the cells of the patient to repair the periodontal defect have been proposed in a number of studies [86-88]. PDL-derived stem cells such as PDLSCs can differentiate into all of the periodontal lineages that contribute to cell turnover in the steady-state and would thus be useful cell sources for regenerative therapies to treat periodontal disease following tissue injury [89-91]. Treatments that partially regenerate damaged PDLs through the local application of cytokines have now been established, and such regenerative therapies have provided a very useful and feasible clinical study model for the future design of stem cell and cytokine therapies [15,61,92]. Although partial regeneration of the periodontal tissue has been established, methods to achieve the functional regeneration of large defects caused by severe periodontal disease are still lacking. To address this, it is essential to better understand the molecular mechanisms underlying PDL development and to thereby identify the appropriate functional molecules that induce the differentiation of stem cells into periodontal lineage cells for the successful reconstruction of periodontal tissue [31,32]. Investigations of the molecular mechanisms of fibrillin-1 microfibril assembly via ADAMTSL6β during

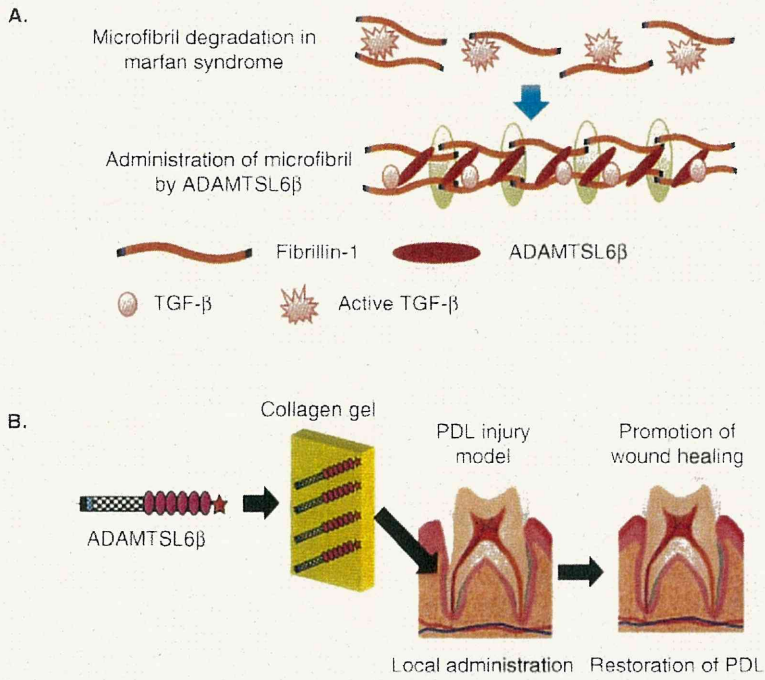

Figure 4. Microfibril administration by administration of ADAMTSL6β. A. Administration of microfibrils by ADAMTSL6β An ECM administration therapy that induces restoration of properly formed microfibrils via ADAMTSL6β is essential not only for improvement of the microfibril disorder, which is a predominant symptom of MFS, but also for the suppression of excessive TGF-β signaling induced by microfibril disassembly. **B.** ADAMTSL6β promotes wound healing of the PDL. A collagen gel containing recombinant ADAMTSL6β is prepared and locally administrated to the injured PDL of Marfan's syndrome mice established via the gene targeting of *fibrillin-1*. In this model, recombinant ADAMTSL6β restores fibrillin-1 microfibril assembly and enhances wound healing.

PDL formation will make substantial contributions to this endeavor [23]. In addition, since microfibrils play an important role in maintaining connective tissue integrity, including the aorta, lung and skin, we are hopeful the ECM administration therapy will in the future encourage the development of PDL regeneration for the treatment of periodontal disease as well as connective tissue disorders such as MFS [75,77].

6. Expert opinion

As described above, the partial regeneration of connective tissue damaged by pathological microflora has been achieved by regeneration therapy using stem cell transplantation and the local application of cytokines. Identification of the stem cells in the PDL or DF has enabled the development of protocols to regenerate the PDL and these have proved to be useful model systems for the development of connective tissue regeneration therapies [15,17]. One of the major research obstacles in PDL regeneration studies is the identification of all of the key functional molecules that drive PDL development. The establishment of ECM administration therapy such as fibrillin-1 microfibril assembly is ultimately critical for the development of new therapeutic approaches for periodontal disease and MFS [76]. MFS fibrillinopathies have been explained by the structural insufficiency of fibrillin-1 microfibrils leading to the activation of TGF-β and its regulatory targets [93]. A variety of MFS therapies have been developed to date, including surgical therapy for aortic root aneurysms that are life-threatening [76], traditional medical therapies such as β-adrenergic receptor blockade for slow aortic growth and to decrease the risk of aortic dissection, and novel approaches based on new insights such as the pathogenesis of insufficient fibrillin-1 microfibril formation and the deregulation of TGF-β activation [77]. In the case of periodontal disease in MFS, surgical therapy or regeneration therapy is performed using stem cells or cytokines to recover damaged periodontal tissue (**Figure 5**, left panel).

In contrast to these approaches, the administration of ADAMTSL6β to fibrillin-1 microfibrils may represent a new ECM administration therapy which is viable for the treatment of the periodontal disease of MFS [23]. The evidence indicates that ADAMTSL6β is capable of enhancing microfibrils even in the case of a fibrillin-1 haploinsufficiency. Hence, ECM

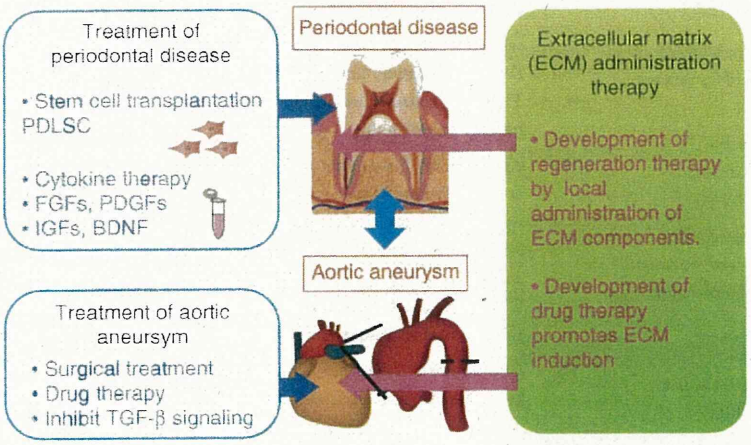

Figure 5. Extracellular matrix (ECM) administration therapy as a novel therapeutic strategy for MFS syndrome. Left panel: A variety of MFS therapies have been developed, including surgical therapy for aortic root aneurysms, traditional medical therapies and mechanisms to deregulate TGF-β activation and thereby decrease the risk of aortic dissection. In the case of periodontal disease, regeneration therapies including stem cell transplantation and cytokine therapy are being performed for the treatment of periodontal disease. Right panel: ECM administration therapy such as ADAMTSL6β administration which induces microfibril assembly should be considered in the development of future mechanism-based therapeutics for the improvement of periodontal disease in MFS. It will also be beneficial to develop drug therapies that promote ADAMTSL6β expression for the treatment of aortic aneurysms.

administration therapy through the promotion of microfibril assembly by ADAMTSL6β may have potentially novel therapeutic benefits for the treatment of periodontal disease and disorders associated with MFS (Figure 5, right panel).

In conclusion, we here introduce the concept that a fibrillin-1-associated protein such as ADAMTSL6β, which induces microfibril assembly, should be considered as an ECM administration agent for the treatment of periodontal disease and improvement of connective tissue disorders such as MFS. The exogenous application of recombinant ADAMTSL6β improves fibrillin-1 microfibril assembly, indicating that the reinforcement of fibrillin-1 microfibrils by ADAMTSL6β may represent a new treatment for periodontal disease which is accessible from oral cavity in MFS patients. Since elastolysis occurs continuously in aortic aneurysms arising in MFS cases, the chronic administration of ADAMTSL6β may be required for the stabilization of microfibrils to prevent progressive tissue destruction. It will also be necessary to develop methodologies for the systemic administration of ADAMTSL6β to induce fibrillin-1 microfibril assembly in connective tissue for the treatment of life-threatening conditions such as an aortic aneurysm (Figure 5, right panel). Hence, an ECM administration therapy involving ADAMTSL6β has the capacity to facilitate drug discovery for treating periodontal diseases, and MFS-associated disorders.

Declaration of interest

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Affiliation

Masahiro Saito[1] DDS PhD & Takashi Tsuji[2]
[1]Author for correspondence
[1]Tokyo University of Science,
Faculty of Industrial Science and Technology,
2641 Yamazaki, Noda, 278-8510, Japan
[2]Tokyo University of Science,
Research Institute for Science and Technology,
Yamazaki 2641, Noda, 278-8510, Japan

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Review Article

Intradermal injection of Botulinum toxin type A alleviates infraorbital nerve constriction-induced thermal hyperalgesia in an operant assay

A. KUMADA*, Y. MATSUKA*, I. SPIGELMAN†, K. MARUHAMA*, Y. YAMAMOTO‡, J. K. NEUBERT§, T. A. NOLAN§, K. WATANABE*, K. MAEKAWA*, H. KAMIOKA¶, T. YAMASHIRO¶, T. KUBOKI* & K. OGUMA‡ *Department of Oral Rehabilitation and Regenerative Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan, †Division of Oral Biology & Medicine, School of Dentistry, University of California, Los Angeles, CA, USA, ‡Department of Bacteriology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan, §College of Dentistry Department of Orthodontics, University of Florida, Gainesville, FL, USA and ¶Department of Orthodontics and Dentofacial Orthopedics, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan

SUMMARY **Recent studies have shown that infraorbital nerve constriction (IoNC)-induced mechanical allodynia has been attenuated by administration of highly purified 150-kDa Botulinum neurotoxin type A (BoNT/A). Here, we extend these studies to determine whether BoNT/A could attenuate IoNC-induced symptoms of thermal hyperalgesia. Instead of testing head withdrawal thresholds, a thermal operant assay was used to evaluate cortical processing of sensory input following IoNC. In this assay, a fasted rat's desire to obtain a food reward (sweetened condensed milk) is coupled to its ability to tolerate facial contact with a warm (45 °C) thermode. Bilateral IoNC decreased the ratio of thermode contact duration/event, which is an indicative of thermal hyperalgesia. BoNT/A injection intradermally in the area of infraorbital nerve (IoN) innervation 7 days after IoNC resulted in decreased number of facial contacts and increased the ratio of contact duration/event (measured at 14 days after IoNC). The BoNT/A (2–200 pg) effects were dose dependent and statistically significant at 100 and 200 pg ($P < 0.05$). Complete reversal of thermal hyperalgesia symptoms was obtained with a 200-pg dose, without affecting sham rat behaviour. Off-site (neck) injection of BoNT/A did not relieve thermal hyperalgesia, while co-injection of BoNT/A with a neutralising antibody in the area of IoN innervation prevented relief of thermal hyperalgesia. Neither IoNC nor BoNT/A injection affected operant assay parameters with a 24 °C thermode, indicating selectivity of thermal hyperalgesia measurements. These results strongly suggest that intradermal injection of BoNT/A in the area of IoN innervation alleviates IoNC-induced thermal hyperalgesia in an operant assay.**

KEYWORDS: **Botulinum toxin, thermal hyperalgesia, trigeminal ganglion, thermal stimulation**

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Introduction

Orofacial neuropathic pain patients often present with symptoms of (in decreasing order of prevalence): spontaneous pain, abnormal mechanical sensitivity (most often dynamic allodynia), cold allodynia and hyperalgesia and heat hypersensitivity (1, 2). Various rodent models of peripheral nerve injury have been developed, which mimic human neuropathy symptoms of tactile allodynia, thermal hyperalgesia and spontaneous pain (3–7). Measuring these symptoms in animal neuropathy models designed to develop the much

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needed new therapies is a challenge, with nociceptive withdrawal thresholds from mechanical or thermal stimulus being the most common measures of symptomatology. Assessment of trigeminal nerve-mediated nociceptive responses has been limited to a handful of methods that assess processing within the brain stem (e.g. withdrawal responses or grooming) (7–10) elicited using Von Frey filaments (7) or thermal stimulation (11). Non-operant assessments of innate behaviours do not reveal cerebral processing of nociception. In this study, we utilised a thermal operant facial testing system to evaluate cortical input following infraorbital nerve constriction (IoNC) (12).

Our research goal is to develop new treatments that decrease chronic pain while minimising side effects. One strategy is to target peripheral sensory neurons whose excitability and neurotransmitter release is increased in chronic pain states (13). Such targeted treatments, which do not penetrate the blood–brain-barrier, should theoretically limit central side effects. One such treatment is Botulinum toxin (BoNT), which reportedly blocks vesicular neurotransmitter release by disabling the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex proteins which mediate vesicular transmitter release (14, 15). Indeed, clinicians have used BoNT, outside the product licence, to treat chronic pain symptoms not associated with muscle spasms (16). Previous studies demonstrated that BoNT is capable of attenuating release of substance P, calcitonin gene-related peptide (CGRP) or glutamate from sensory neurons in culture (17–20), in isolated preparations (21) and *in vivo* (22). We recently reported that IoNC-induced tactile allodynia and vesicular transmitter release from trigeminal ganglion (TRG) neurons were both reduced after peripheral injection of purified Botulinum neurotoxin type A (BoNT/A) in the area of IoN innervation (23). Here, we report that IoNC in rats produces thermal hyperalgesia in an operant assay and demonstrates that peripheral injection of BoNT/A (2–200 pg) dose dependently alleviates the thermal hyperalgesia of IoNC.

Materials and methods

Trigeminal neuropathy model

All procedures regarding animal usage in this study were performed in accordance with specifications of an animal protocol approved by Okayama University (OKU-2009091). Male Sprague Dawley rats (200–300 g) ($n = 28$) were anesthetised by intraperitoneal injection with ketamine (35 mg kg^{-1}) and xylazine (5 mg kg^{-1}), the infraorbital branch of the trigeminal nerve was exposed bilaterally and two silk ligatures (4–0) were loosely tied around the IoN at about 2 mm apart (7, 23). To obtain the desired degree of constriction, a criterion formulated by Bennett and Xie (3) was applied: The ligations reduced the diameter of the nerve by a just noticeable amount and retarded but did not occlude the circulation through the superficial vasculature. The skin incision was closed in layers using nylon sutures (4–0). Control rats were subjected to sham surgery, where the IoN was exposed using the above procedures, but not constricted.

Thermal operant testing

The rats were lightly anesthetised using sevoflurane (2·5%, inhalation), and their hair was bilaterally removed from the orofacial region using clippers, followed by depilatory cream 1 day prior to behavioural testing (12). Excess cream was removed with a moistened paper towel to minimise skin irritation. The rats were food and water fasted for 24 h prior to each testing session, and following each session provided with standard food chow and water *ad libitum*. The animals were brought into the behavioural procedure room 1 h prior to testing and allowed to acclimate to the temperature and ambient noise of the room.

Facial testing was completed using a reward-conflict operant testing paradigm as described previously (12, 24). Briefly, a testing cage (20·3 cm W × 20·3 cm D × 16·2 cm H) with acrylic walls was constructed with an opening in one wall (4 × 6 cm), which was lined with grounded metal (aluminium) tubing. The testing cage is custom made by one of the authors (JKN). The tubing served as a thermode when connected to a water pump (NCB-1200*) via flexible polyethylene tubing through which heated water (45 °C) or non-heated water (24 °C) as control was circulated. Stimulus thermode temperature was verified using a thermometer (Fluke 54†). A standard rodent watering bottle containing a diluted (1:2 with water) sweetened condensed milk solution‡ was mounted outside the

*Tokyo Rikakikai, Tokyo, Japan.
†Fluke, Everett, WA, USA.
‡Morinaga milk, Tokyo, Japan.

cage. The bottle was then positioned in proximity to the cage such that the animal could access to the bottle when simultaneously contacting the thermode with its face. The room temperature was maintained at 24 ± 1 °C for all behavioural tests. Unrestrained animals were placed separately in a testing cage, and the data acquisition system was activated (DI-148U/HS[§]). When the rat drank from the water bottle, the skin on its shaved face contacted the grounded thermode completing an electrical circuit (130 V). The closed circuit was registered in the computer, and data were collected at 60 Hz for the entire length of the experiment. The circuit was established from the metal thermode to the animal by grounding the floor with an aluminium sheet for recording of 'facial contact' events. The duration of each facial contact and the total number of events (facial contact) were recorded. The investigator monitored online data acquisition to ensure each recorded facial contact on the tubing.

Rats were trained twice a week for 2 weeks. Each 10-min training session was performed with the thermode at room temperature. Once trained, the facial testing region for each animal was depilated under light sevoflurane anaesthesia (2·5%, inhalation) once a week to maximise thermal stimulus contact. Baseline measurement began 2–3 days after the training and was performed three times every 3–4 days. Testing was repeated at 7 and 14 days post-surgery and data analysed offline.

During data analysis, the threshold for facial contact detection was set at 1·0 V, above background noise, to minimise false-positive event registration and events typically registered as >5·0 V. A facial contact event was registered when the signal went above threshold and ended when the signal dropped below threshold. The cumulative duration and frequency of events were determined for the facial stimulus contact data. The total amount of milk consumed (g) was measured and compared at each of the testing temperatures. Data were analysed using custom-written routines in LabView Express[¶] and Excel**.

Botulinum neurotoxin injection

After behavioural testing on post-operative day 7, the rats were anesthetised by i.p. injection with ketamine (35 mg kg^{-1}) and xylazine (5 mg kg^{-1}). The BoNT/A was purified by one of the authors (KO) (25, 26). Purified BoNT/A composed of a light and a heavy chain was administered as a single intradermal injection (0, 1, 50 or 100 pg in 0·1 mL of sterile saline) bilaterally [total dose of BoNT/A was 0, 2, 100 or 200 pg, 200-pg dose is equal to 20 units (20 MLD)] in the snout at the centre of the whisker pad (i.e. between rows B and C of the vibrissae) (n = 4 in each dose) or back of the neck of the anesthetised rats (n = 4) with Hamilton syringe (80330, 10 µL, 28 s/s''/s[††]). The bleb of the injection materials was absorbed within a day. In some rats, a mixture of BoNT/A 100 pg and polyclonal anti-BoNT/A antibody 150 ng was injected into the whisker pad (n = 4). The BoNT/A antibody was also generated by one of the authors (KO). It was generated by immunising rabbits with formalin-inactivated BoNT/A and then partially purified by ammonium sulphate precipitation followed by DEAE anion-exchange chromatography. The antibody is polyclonal, and the antibody specificity was determined by enzyme-linked immunosorbent assay (ELISA) and Western blotting methods (Fig. 1). The detail methods of ELISA and Western blotting were same as previous report (26). Thermal testing was repeated on post-operative day 14 (7 days after injection). The control group of sham rats was injected saline with intradermally.

Statistical analysis

Results are presented as group means ± s.e.m. Differences in group means of sham control without BoNT/A injection (Fig. 2) were evaluated by one-way RM ANOVA with Fisher *post hoc* test. Differences between baseline and IoNC (Fig. 3) and between IoNC and BoNT/A with the 24 °C thermode (Fig. 5) were evaluated by paired t-test. Also, differences between IoNC and BoNT/A injection (Fig. 4) were evaluated by two-way RM ANOVA with Fisher *post hoc* test. The Sigma Stat 3·11 software[‡‡] was used for these analyses.

The behaviour of sham surgery rats with the 45 °C thermode changed during the study period, and all data for IoNC rats were divided by the sham rat average data at the same time point. In this study, $P < 0·05$ was considered statistically significant.

[§]DATAQ Instruments, Inc, Akron, OH, USA.
[¶]National Instruments Corporation, Austin, TX, USA.
**Microsoft, Redmond, WA, USA.
[††]Hamilton Co., Reno, NV, USA.
[‡‡]Systat, San Jose, CA, USA.

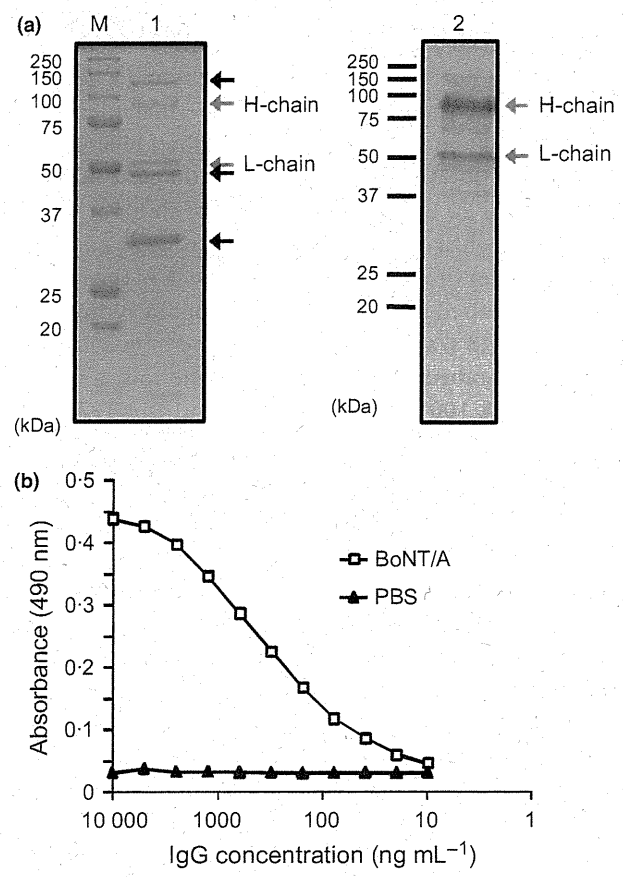

Fig. 1. Anti-Botulinum neurotoxin type A (BoNT/A) antibody specificity with Western blot and ELISA. (a) The progenitor toxin that is a complex of BoNT/A and non-toxic components was separated by SDS-PAGE and then subjected to Coomassie brilliant blue staining (lane 1). The Western blot analysis of the progenitor toxin and anti-BoNT/A antibody showed that the antibody specifically detected the heavy and light chains of BoNT/A (lane 2). Lane M is molecular size marker. (b) ELISA was performed using BoNT/A and anti-BoNT/A antibody serially diluted in twofold step. The ELISA data showed that the titration curve was dose dependent and anti-BoNT/A antibody binds to BoNT/A.

Results

Control rat behaviour in the operant assay

With each test session, sham surgery rats ($n = 4$) showed decreases in the number of contacts with the 45 °C thermode (Fig. 2a). Total contact time per session did not change during the study period (Fig. 2b), while the average duration of each contact increased (Fig. 2c). The behaviour of a separate group of sham surgery rats ($n = 4$) tested with the 24 °C thermode did not change (Fig 2d–f), suggesting that the behavioural changes were specific to the 45 °C thermode.

Effects of IoNC on operant outcome measures

Compared with baseline, measurements obtained at 7 days post-IoNC revealed a trend towards increased number of contact events (Fig. 3a) without changes in the total duration of contacts (Fig. 3b). However, the ratio of contact duration/contact number was significantly decreased after IoNC compared to the baseline data ($P < 0{\cdot}01$) (Fig. 3c). In previous studies with this thermal operant system, this ratio was shown to be a sensitive indicator of thermal hyperalgesia (12). Thus, IoNC rats increased the frequency of the 45 °C thermode contacts while decreasing the duration of each contact ($n = 16$). By contrast, no such changes were seen with another group of IoNC rats tested with the 24 °C thermode ($n = 4$) (Fig 3d–f), while the ratio of contact duration/contact number with the 24 °C thermode increased ($P = 0{\cdot}107$, sample size estimation $n = 10$) (Fig. 3f). The increase in the ratio of contact duration/contact number indicates no thermal hyperalgesia or no tactile allodynia with the 24 °C thermode. IoNC had no effect on total milk consumption in either the sham the IoNC 45 °C or the 24 °C thermode group (data not shown).

BoNT/A reverses the IoNC-induced changes in operant measures

BoNT/A administration reversed the effect of IoNC in the 45 °C thermal operant assay, as it decreased contact event numbers ($n = 4$ in each dose) ($P < 0{\cdot}05$ with 100 and 200 pg BoNT/A to the whisker pad injection) (Fig. 4a) without concomitant changes in the average total contact duration ($n = 4$ in each dose) (Fig. 4b). In effect, this increased the ratio of contact duration/contact numbers ($n = 4$ in each dose) ($P < 0{\cdot}01$ with 200 pg BoNT/A to the whisker pad injection) (Fig. 4c). Moreover, the BoNT/A effects were dose dependent, becoming statistically significant with the 200-pg doses. By contrast, BoNT/A injection had no discernable behavioural effects in the sham-operated rats ($n = 4$) (Fig. 4).

In a separate group of IoNC rats, intradermal BoNT/A injection at the back of the neck did not alleviate thermal hyperalgesia symptoms ($n = 4$) (Fig. 4),

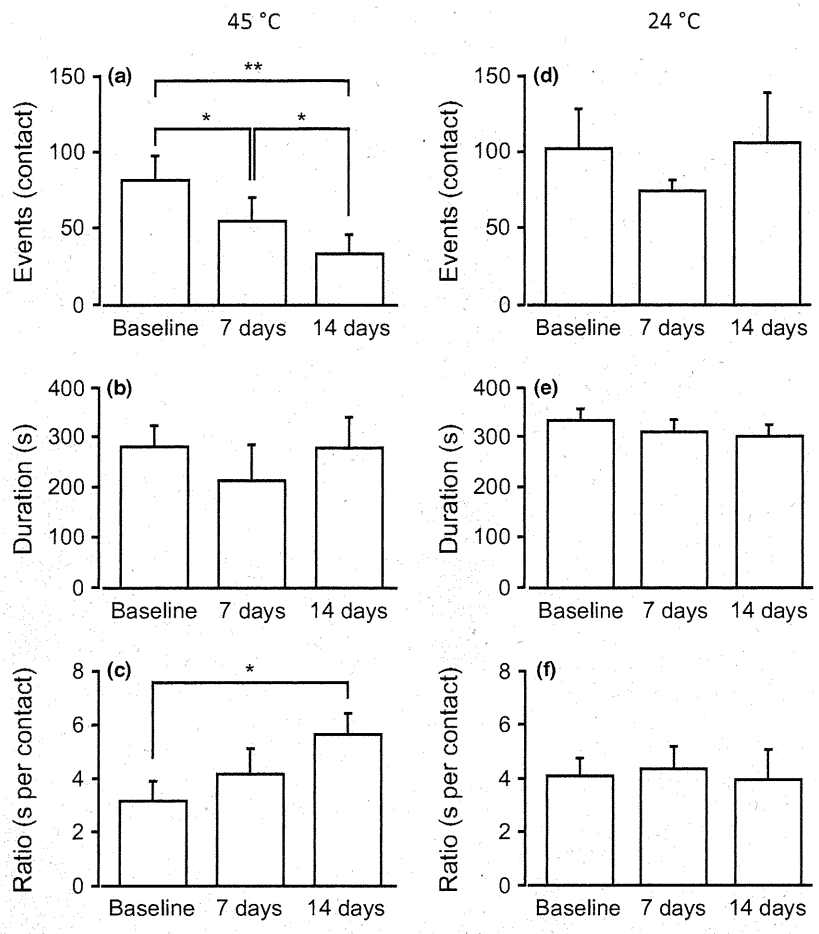

Fig. 2. Effects of sham surgery on operant outcome measures. In rats with the sham surgery ($n = 4$), the contact events at the 45 °C thermode appeared to decrease with each experimental session (a). The total contact time at each session did not change during the study period (b). The average contact duration (ratio of contact duration/contact number) appeared to increase with each experimental session (c). As the sham rat data were altered during the study period, subsequent data for IoNC rats were normalised to the sham rat data at the same time point. The sham surgery rats ($n = 4$) tested with the 24 °C thermode did not change (d–f), suggesting that the behavioural changes were specific to the 45 °C thermode. *$P < 0.05$, **$P < 0.01$, one-way RM ANOVA with Fisher *post hoc* test.

indicating specificity of BoNT/A actions for the innervation area of injured IoN neurons. Another group of IoNC rats was tested in the 45 °C thermal operant assay to address the selectivity of BoNT/A action. To do this, a mixture of BoNT/A and its neutralising antibody was injected bilaterally in the whisker pads ($n = 4$). This injection did not alleviate thermal hyperalgesia symptoms (Fig. 4), demonstrating selectivity of BoNT/A on decreasing the symptoms of thermal hyperalgesia.

Another set of IoNC rats that were previously tested with the 24 °C thermode (Fig. 3) was also treated with BoNT/A (200 pg) to reveal possible BoNT effects on the operant behaviours at the 24 °C thermode. BoNT/A injection at 7 days post-IoNC had no effect on operant behaviour at the 24 °C thermode ($n = 4$) (Fig. 5). These results provided further support for the specificity of BoNT/A effects on thermal hyperalgesia and the lack of tactile allodynia contribution to the rat operant behaviour when contact is made at the thermode.

Discussion

In this study, we demonstrated that IoNC produces thermal hyperalgesia in the region innervated by the IoN, which is consistent with the previous demonstrations that chronic constriction injury of the IoN produces behavioural alterations indicative of trigeminal neuropathic pain (7, 23, 27–29). Most of

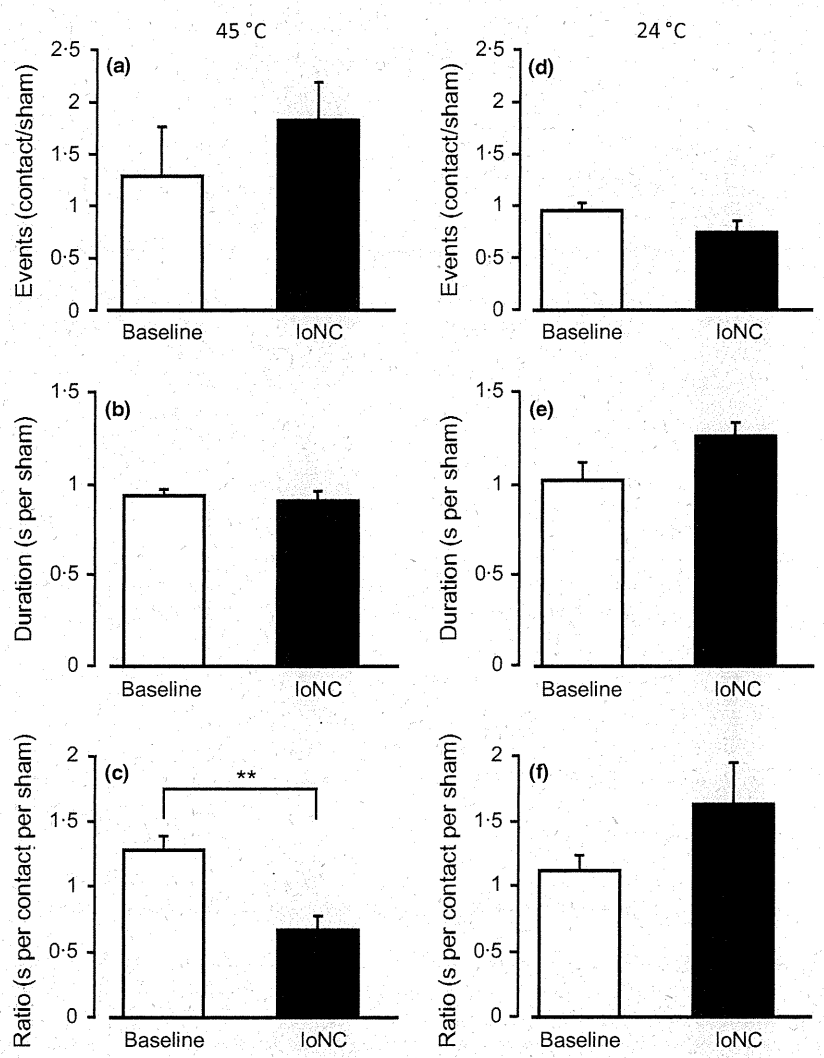

Fig. 3. Effects of IoNC surgery on operant outcome measures. IoNC (n = 16) tended to increase (without reaching statistical significance) the number of contact events at the 45 °C thermode (a). The total contact duration was not changed (b). However, the ratio of contact duration/contact number was significantly decreased (c). The behaviour of sham surgery rats with the 45 °C thermode changed during the study period, and all data for IoNC rats were divided by the sham rat average data at the same time point. By contrast, IoNC had no significant effects on operant behaviour at the 24 °C thermode (d–f). ** $P < 0{\cdot}01$, paired t-test.

these studies measured tactile allodynia (7, 23, 27, 28), and Shinoda *et al.* reported thermal hyperalgesia. Vos *et al.* also reported the observation of free behaviour that indicated pain reaction. However, non-operant assessments evaluate innate behaviours that do not reveal cerebral processing of nociception, and there are few operant models for assessing orofacial pain in rodents (12). Operant responses involve complex behavioural actions and are advantageous in that the animal has control over the amount of nociceptive stimulation and can modify its behaviour based on cerebral processing (30, 31). Conflict paradigms involve learned operant behaviours that reflect animals' choices between receiving a positive reward and escaping aversive stimuli (32). In this study, we evaluated thermal hyperalgesia following IoNC using the thermal facial operant testing system.

Interestingly, the control group of the sham rats showed that the number of contacts with the 45 °C thermode was decreased and the average duration of each contact increased. The behaviour of a separate group of sham surgery rats tested with the 24 °C

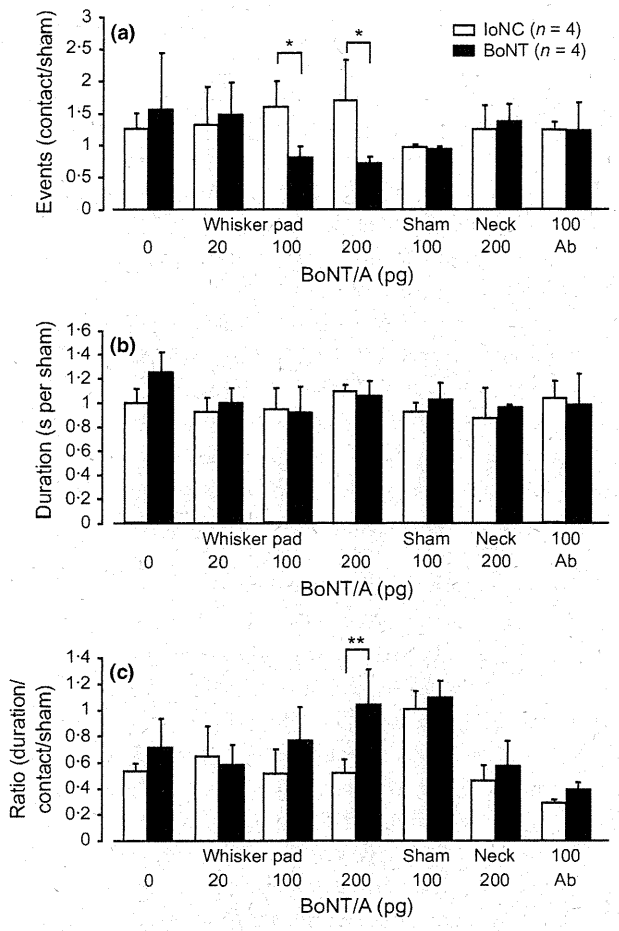

Fig. 4. Botulinum neurotoxin type A (BoNT/A) alleviates IoNC-induced changes in operant measures of thermal hyperalgesia. Intradermal BoNT/A injection in IoNC rats decreased the number of facial contacts at the 45 °C thermode (a) and increased the ratio of contact duration/contact numbers (c). The total contact duration was not affected by BoNT/A (b). The behaviour of sham surgery rats with the 45 °C thermode changed during the study period, and all data for IoNC rats were divided by the sham rat average data at the same time point. The BoNT/A effect was dose dependent, becoming statistically significant with 100 and 200 pg. Sham operation did not affect the rat drinking behaviour, and BoNT/A (100 pg) injection had no discernable behavioural effects in the sham-operated rats. Intradermal BoNT/A injection in the neck, within the trigeminal innervation, but outside of IoN innervation, did not relieve thermal hyperalgesia symptoms. Moreover, injection of a mixture of BoNT/A and its neutralising antibody did not relieve the thermal hyperalgesia. Data are presented as mean + s.e.m. $*P < 0{\cdot}05$ and $**P < 0{\cdot}01$ between the time point of 7 days after IoNC and 7 days after BoNT/A injection.

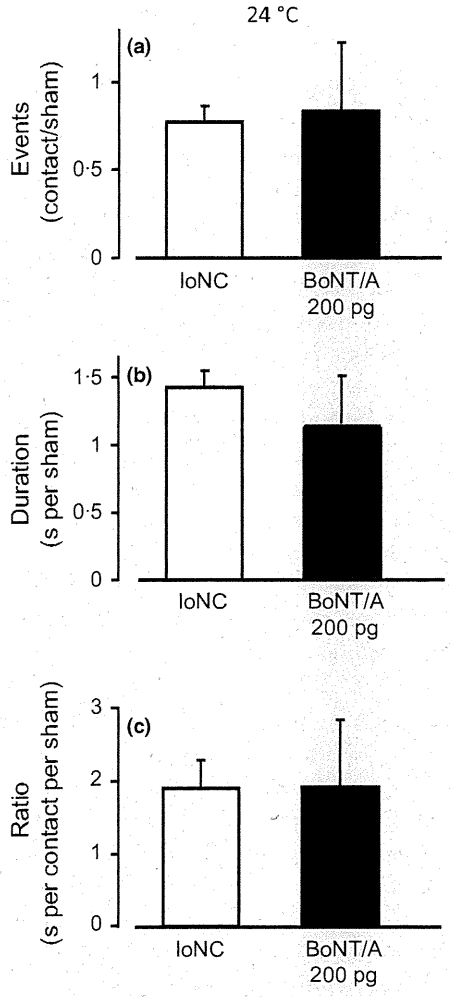

Fig. 5. Botulinum neurotoxin type A (BoNT/A) injection in IoNC rats does not affect operant behaviour with the 24 °C thermode. The IoNC rats do not show significant alterations in the three measured behavioural parameters (a–c) with the 24 °C thermode after BoNT/A (200 pg) injection in the whisker pads ($n = 4$). The behaviour of sham surgery rats with the 45 °C thermode changed during the study period, and all data for IoNC rats were divided by the sham rat average data at the same time point.

thermode did not show such change. Although these phenomena might be related to habituation or learning/memory, we would like to continue to obtain data from control rats with hot, cold or room temperature stimulation for long duration in the future study.

Neubert *et al.* (12) showed that increases in thermode temperature increased the number of facial contacts resulting in more frequent short drinks and that inflammation exaggerated these effects. This present study showed that peripheral nerve injury produced analogous effects in the operant thermal assay, in that the ratio of contact duration/contact numbers was

significantly decreased after IoNC compared with the sham surgery (Fig. 3). Importantly, we demonstrated that at the highest dose (200 pg) BoNT/A completely reversed thermal hyperalgesia symptoms, without affecting the behaviour of sham surgery rats in the thermal operant assay. The result shows the possibility that the BoNT/A reverses the pain symptoms of trigeminal neuropathic pain patients. As the 200-pg BoNT/A dose (20 MLD) is considered a little much for rats, we need to test dose response for trigeminal neuropathic pain patients in the future clinical study.

The IoNC rats did not show hyperalgesia with the 24 °C thermode, and BoNT/A injection did not change their drinking behaviour. This demonstrates that the behavioural changes with the 45 °C thermode were specific to thermal hyperalgesia and not mechanical allodynia. Although we did not record the licking contacts, we found that rats touched the thermode only when they obtained the milk reward, and total reward consumption was not affected by IoNC or BoNT/A treatment.

Previously, we measured tactile allodynia after IoNC and demonstrated that a 100-pg facial BoNT/A injection reversed the allodynia symptoms by ~44% (23). Current thermal hyperalgesia data showed that 50- and 100-pg/side BoNT/A injection reversed the thermal hyperalgesia by 80 and 100%, respectively, suggesting that BoNT/A treatment is more effective at reversing thermal hyperalgesia than tactile allodynia. Neuropathic tactile allodynia may be mediated in part by the activation of the myelinated Aβ-fibres, whereas thermal hyperalgesia is mediated largely by the thinly myelinated Aδ and unmyelinated C-fibre nociceptors. The presence of myelin as well as the specialised low-threshold mechanoreceptors (e.g. Meissner's corpuscles and Merkel disc receptors) on the encapsulated terminals of Aβ-fibres may impede efficient vesicular uptake of BoNT/A resulting in decreased effectiveness of BoNT/A injection at decreasing tactile allodynia symptoms. However, our previous experiments utilised restrained rats to measure tactile allodynia; restraint stress could also account for the differential effectiveness of BoNT/A treatment.

It was reported that responses to cold stimuli in operant assays were more robust than responses to heat stimuli in the bilateral chronic sciatic nerve constriction model (33, 34). It was also reported that male rats in the facial operant assay preferred to contact the 48 °C thermode than the 4 °C, despite the fact that 48 °C and 4 °C were equally painful in the operant assay (24). It will be important to determine the cold stimuli to the IoNC rats and the effect of BoNT/A injection in future studies, as complaints of cold hyperalgesia are more prevalent than thermal hyperalgesia in neuropathic pain patients (1). It would also be important to compare the effects of BoNT/A with other drugs currently used in the clinic (e.g. carbamazepine or gabapentin).

Here, we demonstrated that injection of a mixture of BoNT/A with its neutralising antibody did not relieve IoNC-induced thermal hyperalgesia, suggesting selectivity of BoNT/A antihyperalgesic actions. The selectivity of BoNT/A effects was also confirmed by demonstrating that BoNT/A had no effect on sham surgery rat behaviour at the 45 °C thermode. Detailed mechanisms of the effect of BoNT/A on thermal hyperalgesia are unknown. We previously reported increases in vesicular FM4-64 release from isolated TRG neurons ipsilateral to IoNC and demonstrated that whisker pad injection of BoNT/A decreased this exaggerated release from TRG neurons, measured 11 days after BoNT/A injection (23). Therefore, we suggest that BoNT/A decreases symptoms of thermal hyperalgesia by decreasing IoNC-induced increases in transmitter release from trigeminal sensory neurons.

In the current study, BoNT/A injection outside the area of injured IoN innervation (neck) did not relieve the thermal hyperalgesia, suggesting that BoNT/A injected at the whisker pad of IoNC rats acted directly on IoN neurons. As the BoNT/A injected into the neck did not decrease the pain behaviour, we may be able to conclude that the BoNT/A did not decrease the pain behaviour by central mechanisms. If the BoNT/A works by central mechanisms, the BoNT/A injected into the neck could have decreased the pain behaviour. However, we could not clarify the candidate molecule in this study and need the future study to find the molecule and the BoNT/A working place including trigeminal ganglia (23). Others have reported that BoNT undergoes retrograde transport as well as transcytosis at central synapses (35). As we did not study the details of BoNT/A transport, we could not confirm either the ability of retrogradely transported BoNT/A to undergo transcytosis at central synapses or within the TRG. Future studies using retrograde markers should help to resolve this issue. It would also be important to determine the time course of BoNT/A-induced relief of neuropathic pain symptoms.

Conclusions

Our data show that bilateral IoNC in rats produced long-lasting thermal hyperalgesia in an operant assay. We also demonstrated that intradermal injection of BoNT/A alleviates these hyperalgesia symptoms of IoNC. These data suggest that BoNT/A has a possibility to be a therapeutic drug for patients with certain types of neuropathic pain.

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Correspondence: Takuo Kuboki, Professor and Chair, Department of Oral Rehabilitation and Regenerative Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama, 700-8525, Japan.
E-mail: kuboki@md.okayama-u.ac.jp