CGRP accelerates the pathogenesis of neurological heterotopic ossification following spinal cord injury

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ABSTRACT

Background: Interactions between the heterotopic ossification (HO) and the neurological injuries have got lots of interest but still poorly understood. The present study aims to determine the effect of neuroinflammation related calcitonin gene-related protein (CGRP) on HO following spinal cord injury (SCI).

Method: C57Bl6 mice were anesthetized to make an SCI model, together with the ectopic injection of snake venom cardiotoxin (SVC) or CGRP protein into tibialis anterior (TA) muscles. The TA muscles were then harvested for radiologic, histologic, and immunohistology examinations to determine the formation of bone and the expression of CGRP. Fibro/adipogenic progenitors (FAPs) were isolated and cultured. CGRP protein was added into the medium and then detected for chondrogenic markers.

Result: The callus formed following SVC injection and SCI successfully, with much more formed in the group with SCI, as well as an increased level of CGRP. Similarly, the injection of CGRP directly was able to induce the formation of bone-like tissues in vivo. And, the additional CGRP protein was able to induce chondrogenic differentiation as shown in RT-PCT and immunofluorescence, in a dose-dependent manner.

Conclusion: CGRP was able to induce chondrogenic differentiation of FAPs, which may be an important part of Neurological heterotopic ossification developing.

Introduction

Heterotopic ossification (HO) is common in clinic, which is a poorly characterized degenerative disorder with ectopic bone formation in soft tissue that no effective treatment has been developed ever. Neurological heterotopic ossification (NHO) is common in result of traumatic brain injury (TBI) and spinal cord injury (SCI) [1], with the percentage of 5–20% of severe TBI patients and 20–29% of SCI patients [2]. NHO often forms around large joints like the hip, knee and shoulder joints, causing severe pain and deformation [3]. Similarly, an interesting thing is the fractures combined with traumatic brain injury/spinal cord injury often recover much more rapidly than those without, as well as the more formation of ectopic bones in the soft tissues. Similar results can also be found even in cases peripheral nerve injury (such as brachial plexus injury and tibial fracture). Till now, the cellular and molecular mechanisms of the formation of NHO were poorly understood. Surgical excision is the only treatment for NHO; however, the recurrence of NHO cannot be avoided after surgery [4].

The neurological factors may be the basis for the developing of NHO resulting from traumatic injury. The process of NHO formation is initiated by the peripheral injury with a concomitant central/peripheral neuronal injury which stimulates the bone formation at the site of the peripheral injury via endochondral ossification [5]. The injury of the CNS initiates an inflammatory cascade, together with a range of growth factors and cytokines, which stimulates the recruit and transfer of stem-like cells and finally chondrogenic differentiation at the peripheral injury sites [6]. The neuroinflammation after neural injury introduces the release of the pain mediators like substance P (sP) and calcitonin gene-related protein (CGRP). Substance P is able to initiate expression of almost all known immunological chemical messengers or cytokines [7], as well as specifically recruit of mast cells. Till now, a lot of researches have linked the CGRP with ectopic bone formation [8–10]; however, the mechanism how CGRP involves in NHO remains unknown. CGRP, released by peripheral sensory nerves, was also reported to cause neurogenic vasodilatation, mast cell degranulation, cytokine release, and vascularization [11].

Also, CGRP has been shown to interact with the BMPs, particularly BMP2 and BMP4, two of the main BMPs involved in HO [9,12]. CGRP mediates its effects through both the receptors named calcitonin receptor-like receptor (CALCRL) and activity-modifying protein (RAMP1) [13], which are found throughout the body [14]. The present study aims to investigate the role of CGRP in heterotopic ossification following spinal cord injury.
Method

Animal procedures

The C57Bl6 mice were purchased from Shanghai Slaccas Animal center. All the procedures were approved by the handled as ethics committee of the hospital. The animal procedures were performed according to a previous published literature [15]. Generally, mice were anaesthetized with isoflurane gas and placed in prone position. Skin incision was made on top of thoracic kyphosis to expose the spinal cord. Complete transection of spinal cord was made followed by careful hemostasis. The control group performed only the exposure of the spinal cord but no transection. Tibialis anterior (TA) muscles of the left legs were injured (TAI) by injection 40 ml of snake venom cardiotoxin (10 mM) in both groups. Similarly, to investigate the effect of CGRP in ectopic bone formation, the CGRP (Bachem, Torrance, CA) (10 mM) was injected into the TA muscle of the left leg directly, with the right leg injected with equal volume of PBS for control.

Cell culture

Fibro/adipogenic progenitors (FAPs) were isolated by FACS as described [16]; briefly, hind limb muscles were harvested, minced, and then incubated for 60 min at 37 °C in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco) supplemented with 400 U/ml Collagenase Type II (Sigma). The cell suspension was filtered through 70-µm cell strainers (Falcon) and then centrifuged at 500 × g for 5 min. The cells were then resuspended in FACS buffer and immune-labelled with primary antibodies anti-CD31, anti-CD45, anti-Sca-1, and anti-α7 integrin for 30 min at 4 °C. Cells were then washed and filtered through a 40-µm strainer filter. Flow cytometry analysis and cell sorting were performed on a FACS Aria instrument, and the FAPs were gated based on size, shape, viability, and cell surface markers (CD45-/CD31-/integrin α7-/Sca1 +). Then the cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 20% fetal bovine serum (FBS), 100 mg/ml streptomycin, and 100 U/ml penicillin in a 37 °C_5% CO₂ incubator. When cultures become almost confluent, the cells were treated with 0.5 ml of 0.25% trypsin containing 0.02% EDTA for 2 min at room temperature. The third passage cells were used for further experiments.

For chondrogenic differentiation analysis, 1.0 × 10^5 cells/well was seeded into six-well plates in duplicate. Twenty-four hours after seeding, medium was replaced with chondrogenic medium (CYAGEN) according to the manufactory’s protocol. Cells were then incubated continuously in chondrogenic medium for 14 days. CGRP (Bachem, Torrance, CA) (10, 100, or 1000 ng/ml) was added to the chondrogenic medium.

Radiographic evaluation

The radiographic images were made with the use of CABINET X ray system to evaluate the formation of the callus at 2- and 4-weeks post-surgery. Generally, anaesthetized the mice and put them in prone position. The radiology condition was set at 25 KV and 10 s. Also, at four weeks after modelling, the samples of the legs were collected for evaluation of the cross-sectional area of the callus with the vivaCT 40.

Histology and immunofluorescence

The muscle tissue was isolated, fast frozen in liquid nitrogen, and then embedded in OCT compound. Frozen sections (6 um) were made, mount onto superfrost slides, and then stored at −80 °C refrigerator. Before histology/immunohistochemistry staining, the sections were got out of the refrigerator and fixed in 4% PFA immediately for 15 min. The H&E and Safranin O & staining were performed according to the instructions of the manufacturer.

For immunofluorescence staining, the sections were washed twice with PBS after fixation, treated with PBS containing 0.25% Triton-X for 5 min, blocked with 4% BSA for 30 min in 37 °C, and incubated with primary antibody diluted in 1% BSA for 1 h in 37 °C, followed by washed twice and incubated with proper secondary antibodies. Finally, the slides were incubated with DAPI, dehydrated, and mounted them for further evaluation in microscope.

Quantitative real-time PCR

Total RNA was extracted from tissue samples or cultured cells with Trizol (Qiagen). cDNA was got with PrimeScript™ RT Reagent Kit (Takara) following manufacturer’s protocol. Quantitative real-time PCR (qRT-PCR) was performed using TB Green™ Fast qPCR Mix (Takara) and Applied Biosystems 7500 Real-Time PCR System (Life Technologies) with the standard protocol. All values were obtained triple in a total of three independent evaluations. Calculation for the values was made using the ΔΔCt method.

Western blot

Total protein was extracted from cells using RIPA (Beyotime technology) according to the manufacturer’s protocol. Generally, protein samples were loaded onto and separated using SDS-PAGE, then transferred to PVDF membranes, blocked, and incubated with the primary antibodies at 4 °C overnight. After washing, the membranes were incubated with HRP-conjugated secondary antibodies (Beyotime) for 1 h under room temperature. Immunoreactivity was enhanced using a chemiluminescence kit (Beyotime, Inc, China) and exposed to film. The density of bands on Western blots was quantified using a Bio-Rad image system (Hercules, CA).

Statistical analysis

GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA) was used for analysis of the data. The one-way analysis of variance followed by the Student’s t-test was performed to determine the statistical differences between groups. Results were presented as the mean ± SD, and p values of less than 0.05 were considered statistically significant.
Result

Elevated level of CGRP in HO following spinal cord injury

The X-ray imaging was performed at 2 and 4 weeks after modelling to confirm the formation of callus following injury and SCI (Figure 1A), together with the H&E and Alcian Blue staining (Figure 1B,C). qRT-PCR also demonstrated the formation of chondrogenic and osteogenic callus as the chondrogenic and osteogenic genes were elevated significantly at day 14 (Figure 1D). Also, increased level of CGRP was observed in muscle samples at 14 days after modelling, as shown by qRT-PCR evaluation (Figure 1E).

CGRP promotes callus formation in vivo

Four weeks after the modelling, qualitative radiology examination of the callus showed the trabeculae to be much larger in the SCI group than in the control (Figure 2A). The cross-sectional area of the callus was $25.3 \pm 2.26 \text{ mm}^2$ in the SCI group and $20.3 \pm 2.05 \text{ mm}^2$ in the control group ($p = .003$).

Similarly, the injection of CGRP directly was able to induce the formation of cartilage/bone like tissues as shown by X-ray and histological evaluation (Figure 2B,C), with no such tissue forms in the control group.

CGRP stimulates chondrogenic differentiation of FAPs in vitro

The additional CGRP was able to induce increased levels of chondrogenic markers like Sox9, Aggrecan, and Col II than the control groups, as shown by qRT-PCR of cells at day 7 and 14 days after induction (Figure 3A). Immunofluorescence of Sox 9 also confirmed the ability of CGRP in inducing chondrogenic differentiation (Figure 3B). Moreover, the expression

![Image](https://example.com/image.jpg)

Figure 1. Elevated level of CGRP was observed in HO following spinal cord injury. (A) The X-ray imaging demonstrated the formation of callus 2 and 4 weeks after modelling. (B,C) The H&E and Alcian Blue staining showed the formation of cartilage and bone-like tissues. (D) The chondrogenic and osteogenic genes also elevated significantly at day 14. (E) An increased level of CGRP was observed in muscle samples at 14 days after modelling.
of these genes was also in a dose-dependent manner with the volume of the additional CGRP (Figure 3C).

Discussion

Heterotopic ossification is a disorder involving rapid ectopic bone formation within soft tissues like muscle, tendon, and ligaments, and it has also been linked to neuronal injuries [17]. Here, we determined whether neuroinflammation related mediator calcitonin gene-related protein is involved in NHO. Our results suggest that the CGRP is highly expressed in NHO samples and is also able to promote the chondrogenic differentiation of FAPs, and directly injection of CGRP into muscle is able to induce ectopic bone formation.

FAP is a multipotent progenitor population resident in skeletal muscle; it is useful during development and regeneration, which provides trophic support to myogenic progenitors for muscle fiber maturation and specification [18,19]. Fibro/adipogenic infiltration is a common outcome of chronic muscle disease as seen in elderly population, fibrosis diseases, and myophagism patients; it is also suggested that the FAPs should be responsible for developing it in pathological environments [20,21]. Despite the established importance of FAPs in both regeneration and degeneration, the signals that regulate the choice between these alternative fates are still poorly characterized, as well as the development of ossification. Previous studies have demonstrated that FAPs are a major source of cells for both injury-induced and spontaneous HO [22,23]. Here, we also demonstrated the FAPs can be induced by CGRP for chondrogenic differentiation during the pathologies of neurologic ossification.

CGRP is a neuropeptide released from both the peripheral and the spinal cord terminals of nociceptive neurons in response to various stimulations. CGRP-positive sensory fibers are present in bone which are considered to work as a feedback of fracture pain [24,25]. In the tissues other than bone, CGRP is also known to work as a balance between tissue and sympathetic nervous system. In the bone, CGRP is found able to directly influence the function of osteoclast. In vitro, CGRP could inhibit the formation of osteoclasts from bone-marrow precursors [26]. CGRP has been directly linked to the HO pathophysiology with its function on immunoregulatory, such as immune cell recruitment and cytokine release [27,28]. Also, as known to us all the importance role of BMP signaling in hereditary and acquired HO, CGRP was found to
function along with the BMPs [29]. The neuropeptides up-regulated BMP expression and induced osteogenic differentiation in osteoprogenitor stem cells through up-regulation of cAMP levels [30]. Here, we also demonstrated similar results that besides the role of inflammation regulation; CGRP was also able to induce chondrogenic differentiation of FAPs. However, the molecular basis of it remains to be determined.

Conclusion

NHO is common in clinic with a huge impact on patients’ quality of life. The reason of HO remains unknown, as well as the reliable treatment. To better understand the mechanism of NHO pathology, we developed a spinal cord injury model and found CGRP was able to induce chondrocyte differentiation of FAPs, which may be an important part of NHO developing.

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