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Dural fibroblasts play a potential role in headache pathophysiology

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ABSTRACT

Nociceptive signaling from the meninges is proposed to contribute to many forms of headache. However, the events within the meninges that drive afferent activity are not clear. Meningeal fibroblasts are traditionally thought to produce extracellular proteins that constitute the meninges but not to contribute to headache. The purpose of these studies was to determine whether dural fibroblasts release factors that activate/sensitize dural afferents and produce headache-like behavior in rats. Dura mater was removed from male rats and dural fibroblasts were cultured. Fibroblast cultures were stimulated with vehicle or lipopolysaccharide (LPS), washed, and conditioned media was collected. Fibroblast media conditioned with vehicle or LPS was applied to retrogradely labeled rat dural trigeminal ganglion neurons in vitro. Patch-clamp electrophysiology was performed to determine whether conditioned media activated/sensitized dural afferents. A preclinical behavioral model was used where conditioned media was applied directly to the rat dura to determine the presence of cutaneous facial and hind-paw allodynia. Conditioned media was also tested for interleukin-6 (IL-6) content using an enzyme-linked immunosorbent assay. Application of LPS-conditioned fibroblast media to dural afferents produced a significant increase in action potential firing as well as cutaneous facial and hind-paw allodynia when this media was applied to the dura. Finally, stimulation of cultured fibroblasts with LPS increased IL-6 levels in the media. These findings demonstrate that fibroblasts stimulated with LPS release factors capable of activating/sensitizing dural afferents. Further, they suggest that fibroblasts play a potential role in the pathophysiology of headache.

1. Introduction

The pathophysiology contributing to many forms of headache is not clear. Within the skull, the only pain-sensitive structure is the meninges and the only sensation induced from the meninges is pain [28]. Understanding the mechanisms leading to activation/sensitization of meningeal afferents may provide important clues into the pathophysiology of headache.

Many inflammatory mediators including acidic pH, histamine, bradykinin, prostaglandins, nitric oxide, and serotonin can activate and/or sensitize dural afferents [2,7,14,22,29,33,37], and the presence of these mediators in the meninges may cause headache. Mast cells and macrophages within the meninges have also been proposed to contribute to headache [11,25,26,39], and these cells are a potential source of a variety of inflammatory mediators. However, the primary resident cell type in the meninges is fibroblasts. Dural fibroblasts are elongated cells with extended cell processes that show a fusiform or spindle-like shape and are oriented parallel to the flat axes of the dura mater [13]. These cells are responsible for producing the collagen, fibronectin, and other extracellular matrix proteins that make up the meninges, particularly the dura [31]. In addition to producing the dura, fibroblasts may also play a role in activating/sensitizing dural afferents via the release of proinflammatory substances. However, these cells have not been studied for potential contributions to headache. The purpose of these studies was to investigate whether dural fibroblasts play a potential role in the pathophysiology of headache by examining their release of factors that can activate/sensitize dural afferents.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (35–100 g for dural fibroblast culture, 150–175 g for patch-clamp studies, 250–300 g for behavioral

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studies) were maintained in a climate-controlled room on a 12-hour light/dark cycle with food and water ad libitum. All procedures were performed in accordance with the policies and recommendations of the International Association for the study of Pain, the National Institutes of Health guidelines for handling and use of laboratory animals, and were approved by the Institutional Animal Care and Use Committee of the University of Arizona.

2.2. Surgery

2.2.1. Retrograde tracer injection

Dural afferents were identified as previously described [37]. Briefly, 7 days prior to sacrifice, animals were anesthetized with a combination of ketamine and xylazine (80 mg/kg and 12 mg/kg; Sigma-Aldrich, St. Louis, MO, USA), and 2 holes (3 mm in diameter) were made in the skull. Five microliters of Fluoro-Gold (FG, 4% in synthetic-interstitial fluid; Fluorochrome LLC, Denver, CO, USA) was then applied onto the dura. A small piece of Gelfoam (Pfizer Inc., New York, NY, USA) was retained in the hole to increase absorption of dye and prevent dye spread out of the holes. The holes were covered with bone wax to prevent tracer leakage. Immediately postoperatively, animals received a single subcutaneous injection of gentamicin (8 mg/kg) to minimize infection. Dura at the injection sites was evaluated at the time the animals were sacrificed, and only data from animals with intact dura and no signs of damage were used for further analysis.

2.2.2. Dura cannulation

Dura cannulae were implanted as previously described [37]. Animals were anesthetized with a combination of ketamine and xylazine (80 mg/kg and 12 mg/kg; Sigma-Aldrich). A 2-cm incision was made to expose the skull. A 1-mm hole (above the transverse sinus; 2 mm left of sagittal suture and 2 mm anterior to lambdoid suture) was made with a hand drill (Plastics One, Roanoke, VA, USA) to carefully expose the dura. A guide cannula (Plastics One), designed to extend 0.5 mm from the pedestal to avoid irritation of the dural tissue, was inserted into the hole and sealed into place with glue. Two additional 1-mm holes were made rostrally to the cannula to receive stainless-steel screws (Small Parts, Inc., Logansport, IN, USA), and dental acrylic was used to fix the cannula to the screws. A dummy cannula (Plastics One) was inserted to ensure patency of the guide cannula. Immediately postoperatively, animals received a single subcutaneous injection of gentamicin (8 mg/kg) to minimize infection. Rats were housed separately after surgery and allowed 6–8 days of recovery.

2.3. Cell culture

2.3.1. Trigeminal ganglion culture for electrophysiology

Seven days following Fluoro-Gold application, trigeminal ganglia (TG) were removed, enzymatically treated, and mechanically dissociated, as previously described [35,37]. Rats were anesthetized with isoflurane (Phoenix Pharmaceuticals, Burlingame, CA, USA) and sacrificed by decapitation. The TG were removed and placed in ice-cold Hanks balanced salt solution (divalent free). Ganglia were cut into small pieces and incubated for 25 minutes in 20 U/mL Papain (Worthington Biochemical Corporation, Lakewood NJ, USA) followed by 25 minutes in 3 mg/mL Collagenase Type II (Worthington). Ganglia were then triturated through fire-polished Pasteur pipettes and plated on poly-D-lysine (Becton Dickinson, Franklin Lakes, NJ, USA) and laminin (Sigma)-coated plates. After several hours at room temperature to allow adhesion, cells were cultured in a room-temperature, humidified chamber in Liebovitz L-15 medium supplemented with 10% fetal bovine serum, 10 mM glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 50 U/mL penicillin/streptomycin. Cells were used within 24 hours post plating.

2.3.2. Dural fibroblast culture

Rats were anesthetized with isoflurane and sacrificed by decapitation. The dura mater from 6 animals were removed and placed in ice-cold Hanks balanced salt solution. Dura mater were cut into small pieces and incubated in collagenase A (1 mg/mL; Roche Applied Science, Indianapolis, IN, USA) and collagenase D (1 mg/mL; Roche) with papain (30 U/mL; Roche) for 40 to 50 minutes. To eliminate debris, 70-μm cell strainers (BD Biosciences, San Jose, CA, USA) were used. The dissociated cells were resuspended in Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (Invitrogen, Life Technologies, Grand Island, NY, USA) containing 1× pen-strep (Invitrogen), 1× GlutaMAX (Life Technologies), and 10% fetal bovine serum (HyClone, Thermo Fisher Scientific, Waltham, MA, USA). The cells were plated in one 6-well plate (BD Falcon, BD Biosciences) and incubated at 37°C in a humidified 95% air/5% CO_2 incubator. Cultures were maintained in media until time of treatment. Dural fibroblasts were plated at a density that would achieve confluency by day 3 post plating, and cultures were used at this time point. Cultures were not passaged in any of these experiments.

2.4. Immunocytochemistry

Dural fibroblasts were cultured as described above and placed on Poly-D-Lysine-coated coverslips (BD Biosciences) until 3 days post plating. Three different cultures were generated from 3 different sets of rats. Cells were washed with phosphate-buffered saline (PBS), permeabilized with PBS + 10% normal goat serum + 0.1% Triton X 100, and blocked with PBS + 10% normal goat serum + 0.01% Na-azide. A primary rabbit glial fibrillary acidic protein (GFAP) antibody (1:1000; Cell Signaling, Danvers, MA, USA) or a primary rabbit von Willebrand factor (vWF) antibody (1:200; Abcam, Cambridge, MA, USA) was applied to dural fibroblast cultures for 36 hours. A primary vimentin antibody (1:500; Millipore, Billerica, MA, USA) or primary CD68 antibody (1:200; AbD Serotec, Oxford, UK) was applied to dural fibroblast and incubated overnight. A secondary goat anti-rabbit 488 or goat anti-rabbit 555 antibody (1:2000; Invitrogen) was applied for 1 hour to each group and mounted in Prolong Gold with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) and coverslipped. Slides were visualized using a Zeiss confocal microscope (Carl Zeiss Microscopy, Jena, Germany). Dural fibroblast culture images were taken at 40× and tiled (using Zeiss ZEN) to increase the field of view. Images were histogram stretched with no gamma change. For cell counts (see Results), 3 slides were used for each antibody. From each slide, 5 random fields were chosen and images were captured at 20×. Images were histogram stretched until background staining of fibroblasts was just barely visible, and any staining around the DAPI nuclei that was above background was defined as positive staining. Staining that was not near a DAPI-labeled nucleus was not counted as positive. Total cell counts were performed using the automated DAPI count feature in ImageJ (National Institutes of Health, Bethesda, MD, USA). Cell counting was performed blinded to the antibody conditions.

2.5. Fibroblast-conditioned media collection

Confluent cultures of dural fibroblasts (3 days post plating) were treated with 5 ug/mL of lipopolysaccharide (LPS) from *Escherichia coli* (Sigma L2630) in media (Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 with 1× GlutaMAX) or vehicle (plain media) for 1 hour. After treatment, cells were washed 3 times with media to remove the LPS or vehicle. Next, 600 μL of media was

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