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Effects of medium flow on axon growth with or without nerve growth factor

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ABSTRACT

Axon growth is a crucial process in regeneration of damaged nerves. On the other hand, elongation of nerve fibers in the epidermis has been observed in skin of atopic dermatitis patients. Thus, regulation of nerve fiber extension might be an effective strategy to accelerate nerve regeneration and/or to reduce itching in pruritus dermatosis. We previously demonstrated that neurons and epidermal keratinocytes similarly contain multiple receptors that are activated by various environmental factors, and in particular, keratinocytes are influenced by shear stress. Thus, in the present study, we evaluated the effects of micro-flow of the medium on axon growth in the presence or absence of nerve growth factor (NGF), using cultured dorsal-root-ganglion (DRG) cells. The apparatus, AXIS™, consists of two chambers connected by a set of microgrooves, through which signaling molecules and axons, but not living cells, can pass. When DRG cells were present in chamber 1, NGF was present in chamber 2, and micro-flow was directed from chamber 1 to chamber 2, axon growth was significantly increased compared with other conditions. Acceleration of axon growth in the direction of the micro-flow was also observed in the absence of NGF. These results suggest that local micro-flow might significantly influence axon growth.

1. Introduction

Axon growth is essential for regeneration of damaged nerves [1]. On the other hand, abnormal elongation of axons in the epidermis occurs in skin of atopic dermatitis patients [2,3]. Thus, an understanding of the regulation of axon growth might have clinical relevance. We previously demonstrated that similar series of receptors that respond to various environmental factors are expressed in both neurons and epidermal keratinocytes [4]. Moreover, shear stress influences keratinocytes [5]. Thus, we hypothesized that physical environmental factors might influence axon growth. In the present study, we focused on the effect of micro-flow in the axon environment.

We recently demonstrated that keratinocyte-derived factors influence axon growth by using an apparatus, AXIS™, consisting of two different channels connected via a set of microgrooves through which signaling molecules and axons, but not living cells, can pass [6]. With this apparatus, we can induce micro-flow of the medium through the microgrooves simply by applying more medium in one chamber than in the other chamber. If more medium is applied in the other chamber, the direction of micro-flow is reversed, and if the same amount of medium is applied in both chambers, no micro-flow occurs.

In the present study, we used this apparatus to examine whether axon growth of cultured neurons derived from dorsal-root-ganglion (DRG) is influenced by micro-flow of the medium in the presence or absence of nerve growth factor (NGF).

2. Materials and methods

The present study was approved by the ethics committee of Shiseido Research Center, and was conducted in accordance with the guidelines of the National Institute of Health.

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2.1. Cells and cell culture

Primary rat dorsal-root-ganglion (DRG) cells were purchased from Lonza (Walkersville, MD, USA). DRG cells were seeded in Millipore's Axon Investigation System (AXIS, AX45005PB, Fig. 1A) coated with cell attachment matrix solution (poly-D-lysine, laminin, collagen type IV, poly-L-ornithine). This system has two channels connected via a set of microgrooves, through which signaling molecules and axons, but not living cells, can pass. The height of each microgroove is 5 μm, the width is 10 μm and the length is 450 μm (Fig. 1A). We seeded DRG cells (1×10^4 cells) in one of the two chambers. The culture medium on AXIS was Epilife basal medium supplemented with S7 (Invitrogen, Carlsbad, CA, USA), B27 (Invitrogen), penicillin-streptomycin (Invitrogen) and 1.8 mM Ca^{2+}. The medium was changed every day. In some experiments, β-NGF (human β-NGF; Sigma) was included in the medium (100 ng/ml) in one of the two chambers, beginning one day after seeding DRG; in other experiments, NGF was not added.

At the time of seeding, the volume of the medium was 400 μl in each well (no micro-flow). After 24 h, micro-flow was induced by changing the volume of medium to 400 μl medium in one chamber and 300 μl in the other. To halt micro-flow, we changed the volumes of medium to 400 μl in each chamber. When the volumes of medium in the two chambers differ by 100 μl, the difference in the height of the medium between the two chambers is around 1.1 mm. This difference induces a pressure gradient in the microgrooves, and the average flow speed in the microgrooves was calculated to be 49 μm/s, corresponding to a volume flux of 2.45 pL/s, considering Poiseuille flow.

2.2. Immunohistochemistry

After culture for 4 days, DRG cells grown in the AXIS chamber were fixed with 4% paraformaldehyde. Axons and DRG cells were fixed with monoclonal mouse anti-β-tubulin antibody (1:1000; Sigma, St Louis, USA). DRG cell bodies were stained with Hoechst 33258. As secondary antibody, we used anti-mouse antibody conjugated with Alexa Fluor® 488 (1:1000; Invitrogen).

2.3. Data analysis

Penetration (growth) of axons from the DRG-cell side to the other side was quantified as follows. First, anti-β-tubulin mouse antibody staining (Fig. 1B) visualizes axons and neuron cell bodies, neurons while Hoechst staining (Fig. 1C) visualizes neuron cell bodies. These images were processed to grayscale and then binarized. Fig. 1D shows the binarized image of Fig. 1C. From the binarized image of Fig. 1B, we subtracted the image of Fig. 1D to obtain a binarized image of axons (Fig. 1E). The numbers of white pixels in the binarized image were counted and used as indexes of axon and cell number.

2.4. Statistics

The results are expressed as the mean ± SD. Statistical differences were determined by ANOVA test (Fisher's protected least significant difference).

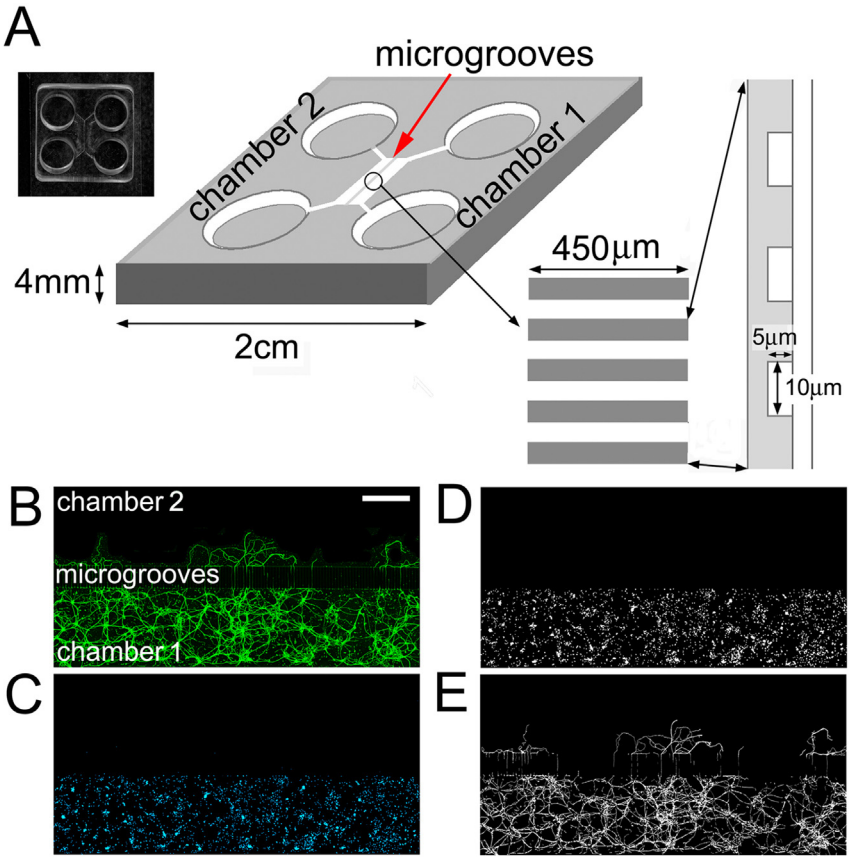

Fig. 1. The experimental system and the process of numerical analysis. A: Structure of Millipore's Axon Investigation System (AXIS, AX45005PB). B: Image of immunostaining by anti-β-tubulin antibody. Bar = 1 mm. C: Image of Hoechst staining. D: Binarized image of C. E: Image of axon obtained by subtraction of images D from binarized image B. All images are of the same region.