行政院國家科學委員會專題研究計畫　成果報告

中性球及其誘引激素在周圍神經瓦勒氏變性的角色

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一、中文摘要

當周圍神經受傷後遠端會產生固定的細胞變化，我們稱為瓦勒氏變性。與中樞神經不同，周圍神經受傷後能夠再生，主要是因巨噬細胞在受傷後迅速增加，他們除了清除會抑制 axon 生長的 myelin 外，刺激 Schwann cell 分裂，還會分泌 IL-1，才能讓非神經細胞分泌神經生長激素。我們研究為何單核球以 MCP-1（Knock-out）mice 作 axotomy 後，巨噬細胞仍然出現，雖然神經 degeneration 的程度稍慢 2-3 天，我們以中性球的抗體（MCA967 , Serotec , UK.），發現在 Axotomy 後 1 至 2 天遠端處（離受傷處 0.5 公分），皆有多量的中性球出現。這種炎症反應在程度及時間上，與一般的炎症反應並無不同。CINC（中性球的 chemokine）mRNA 在第一天及第二天有一小 peak 的表現，約與 MCP-1 的表現同時，故 Block 了下游，並不能完全阻斷這炎症連鎖反應（inflammatory cascades）。當然巨噬細胞的 chemokine 具 redundancy（即還有其他 MCP，如 MCP-2、MCP-3 或 LIF 等）也是因素。

關鍵詞：nerve degeneration; nerve regeneration; peripheral nerves; neutrophils

Abstract

MCP-1 is the major chemokine expressed during Wallerian degeneration for attracting monocytes. However, we observed similar influx of macrophages within the degenerating sciatic nerves of MCP-1 knocked out mice, with a delay of about 2-3d in the extent of degeneration. Besides, there is a small peak of CINC (The chemokine for neutrophils) expression on 1 d post-axotomy. We observed early influx of neutrophils at 1 to 3 d within the Wallerian degeneration models. By double labeling with ED1 and MCA967 (anti-neutrophil ab), we noted that neutrophils come first and fast and are replaced with macrophages later just as in other inflammation processes. Thus we could explain why macrophages still infiltrating within the degenerating sciatic nerve of MCP-1 KO mice. The upstream position of the role that neutrophils play makes the downstream MCP-1 knockout ineffective. Besides, there is a redundancy of monocyte chemokines.

二、Introduction

Outside the nervous system myelomonocytic cells or neutrophils are known to play an important role in the inflammatory response and tissue repair after injury. The early inflammatory cell, the neutrophil, contributes to many diseased states, such as ischemia reperfusion injury, Guillain-Barre syndrome, trauma of peripheral nerves and hyperalgesia. Their appearance during any kind of injury is a common phenomenon, even in Wallerian degeneration, which was classified as macrophage-only degeneration. The function of these endoneurial neutrophils was unknown and never mentioned in literature regarding Wallerian degeneration.

三、Materials and Methods

Male 8-week-old Wistar rats were anesthetized by pentothal i.p. injection (40 mg/kg). Through a 10-mm incision and by blunt dissection of superficial and medial gluteal muscles, the right sciatic nerve was exposed and cut at the sciatic notch under aseptic conditions. After transection, the distal stump of the nerve was diverted into the muscle to minimize regrowth of nerve fibers. At specific time points, deeply anesthetized rats were decapitated and exsanguinated. The mid-thigh portions of sciatic nerves from both sides were harvested, snap-frozen in liquid nitrogen, and kept at -80°C until used. The first segment of sciatic nerves that remained distal to the transection (6-7 mm long) was taken after transcardial fixation with a cold mixture of 2% paraformaldehyde, lysine, and sodium periodate[1]. .sciatic nerve was further fixed in the same fixative for 12-16 h and kept in 0.05 M Tris-buffered saline (TBS). The first 2 mm distal to the transection was excluded from analysis, so that the analysis focused only on the segment 2-7 mm distal to the transection. Four animals were killed at each of the following time points: 3 h, 6 h, 1 d, 2 d, 4 d, 7 d, 14 d, and 21 d after nerve transection. Sciatic nerves from the left side served as controls. All data were representative of three experiments

performed independently. All procedures were approved by the Animal Care Committee of the National Taiwan University College of Medicine.

Effect of neutrophil depletion on Wallerian degeneration

Circulating neutrophils were depleted by i.p.injection of selectively cytotoxic rabbit ani-rat neutrophil antibody (Accurate, USA)[2]. Rats are aneaesthetized . The dose administered was 2 ml/kg (i.p.) for both the antibody and the normal serum, diluted 10-fold in 0.9% sterile saline. The antibody was diluted to avoid possible clumping of lysed neutrophils in the lungs, which could cause death.

RNA extraction

Pooled frozen samples of mid-thigh sciatic nerves (10-mm segments taken from the distal stumps 6-7 mm distal to the site of transection) from four mice were homogenized in a 1.5 ml Eppendorf tube with a Kimble pestle (Fisher; Pittsburgh, PA). Total RNA was obtained by using TRIZOL extraction reagent (Life Technologies; Gaithersburg, MD).

Primers, Probes, and Reference Internal Control mRNA. Primers and probes were chosen using the computer program, Primer Express (Perkin-Elmer Applied Biosystems, Forster City, California). Primers and probes were synthesized and bought from Perkin Elmer Applied Biosystems. Based on the cDNA sequence (Gene bank, CINC), the primers used for RTQ RT-PCR of CINC mRNA were as follows: (a) forward primer, 5'-TTTTGAGAACATCCAGAGCTTGAC-3' and (b) reverse primer: 5'-CTTGAGAGTGGCTATGACTTCTGTC T-3'. The sequence of the probe for detection and quantification of PCR product was as follows: 5'-TGACCCCTCCAGGACCGCACTG-3' (169-190). ß-actin mRNA (internal control) in the nerve sample was quantified in the same way, using forward and reverse primers and a probe designed for ß-actin mRNA analysis. The forward primer sequence for ß-actin mRNA quantification was 5'-TACTGCCCTGGCTCCTAGCA-3'; the reverse primer sequence was 5'-TGGACAGTGAGGCCAGGATAG-3'; and the probe sequence was 5'-AAG ATC ATT GCT CCT CCTGAG CGC AAG T-3'.

RT-PCR Procedure. The amplification mixture (50 µl contained 50 ng of sample RNA, 5X TaqMan EZ buffer (10µl*)*, 25 mM manganese acetate (6 µl), 300 µM deoxyadenosine triphosphate (dATP) deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and 600µM deoxyuridine triphosphate (dUTP), 5 units of rTth DNA polymerase, 0.5 units of AmpErase uracil N-glycosylase (UNG), 200 nM CINC forward and reverse primers, and 100 nM CINC probe (PerkinElmer Applied Biosystems). The rTth DNA polymerase had both reverse transcriptase and Taq polymerase activity. The thermal cycling parameters were as follows: initial step of 2 minutes at 50° C, 30 minutes at 60° C for reverse transcription, 5 minutes at 95° C for deactivation, and then 40 cycles at 94° C for 20 seconds and 62° C for 1 minute for the melting, annealing, and extending phases of the PCR reaction. Each assay included standard curve samples in duplicate, a no-template control, and about 50 ng of sample total RNA in triplicate.

Immunocytochemistry

Ten-µm longitudinal and cross sections of OCT-embedded cryosection were mounted on Poly-lysine-coated glass slides. After drying for 2 h, sections were rehydrated for staining or stored at -80°C for future use. The sections were washed with TBS and treated with 3% hydrogen peroxide in TBS for 30 min, and then incubated for 30 min in 10% normal goat serum (Vector Laboratories; Burlingame, CA) and 1% Triton X-100 (Sigma; St. Louis, MO) in TBS. The slides were then incubated overnight at 4°C with monoclonal antibodies against macrophages

(ED1, Serotec, UK), neutrophil (MCA967, Serotec) and rat CINC (AB1830P,GRO/KC Chemicon, USA). The sections were then washed and incubated with a biotinylated secondary Ab (1: 300,Vector) followed by a streptavidin-biotin-peroxidase solution (Dako; Carpenteria, CA) for 30 min. The slides were developed with diaminobenzidine (Sigma) and either counterstained with Mayer's hematoxylin for single labeling, or processed as follows for double-labeling.
Method-specific tests were done by replacing either one or both of the primary Abs on first or second immunostaining with non-immune rabbit IgG at the same working concentration of primary Ab. Thus, no staining was due to any immunoreagent other than the primary Abs. The double-labeling procedures were checked by reversing the sequence of staining [3].

Estimation of macrophage and neutrophil densities at various time point by double immunostaining

The anti-neutrophil ab (MCA967, Serotec, U.K.) and ED1 ab was sequentially used to double-label the same sections with DAB and SG as brown and dark blue reaction products. We always used DAB first and SG last. The whole immunostaining procedures were reversed to check the blocking effect of DAB reaction product. Method specific test was done by replacing the primary ab with non-immune IgG at the same working concentration of primary ab. Thus no staining was found due to nonspecific action.
A uniform systematic random sampling method[4] was used to count the ED1 or MCA967-positive cells. Sections were viewed with an Olympus BH-2 microscope. Sections were outlined first at a low power (4× objective, magnification: ×400) to delineate the area of quantitation. By applying an unbiased counting frame of known area (6400 μm^2) [5], systematic non-overlapping series of fields were examined across the whole nerve section at a random non-overlapping fashion. Only those ED1 or MCA967-positive cells with counterstained nuclei were counted. To avoid bias, slides were read in blinded fashion, as regards control or experimental animals and day of sacrifice. At least four sections for each animal and four animals from each group were included in this experiment.

四、Results

In rats, the nerve segments distal to the axotomy are in Wallerian degeneration. In intact sciatic nerve, there is no labeling of neutrophil (A). As early as 1 d post-axotomy (B), there are abundant MCA967(+) neutrophils that are very away from the injured zone. By labeling with ED 1 and developed with DAB (brown product) firstly and by labeling the sections secondarily with MCA967 and developed with SG kit (dark blue product). We could see the distribution of these two kinds of leukocytes during Wallerian degeneration. By 4 d post-axotomy (E), there is dramatic decrease of neutrophils and by 5 d (F) almost no neutrophil could be found.

After we found that MCP-1 knockout has minor phenotype in regards to macrophage recruitment and delay in myelin clearance, we looked at the early expression of CINC mRNA which is the chemokine for neutrophils. TheCINC mRNA has a small peak at 2 d post-axotomy. The peak disappears soon. These early inflammatory cells might contribute to the late recruitment of macrophages bypass the MCP-l expression. The other CINC mRNA data was under analysis and was shown very interesting patterns which need further testing and will be shown in the final report.

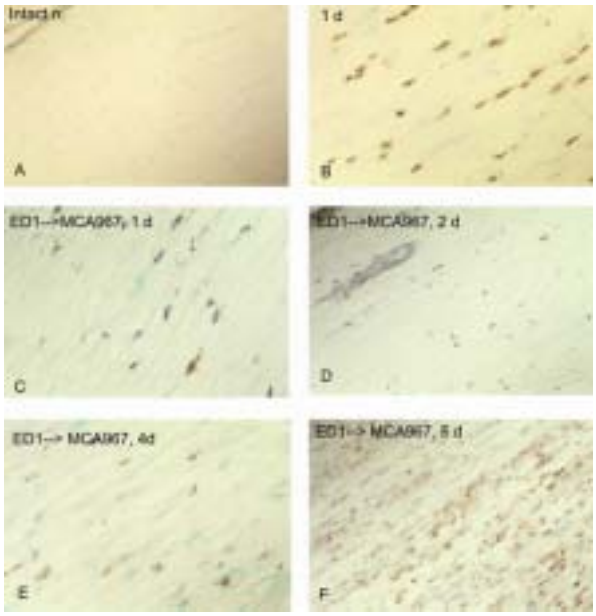

Figure 1. Neutrophils infiltration at early stage of Wallerian degeneration.

四．討論及自我評估

Outside the nervous system myelomonocytic cells or neutrophils are known to play an important role in the inflammatory response and tissue repair after injury. The early inflammatory cell, the neutrophil, contributes to many diseased states, such as ischemia reperfusion injury, Guillain-Barre syndrome, trauma of peripheral nerves and hyperalgesia. Their appearance during any kind of injury is a common phenomenon, even in Wallerian degeneration, which was classified as macrophage-only degeneration. The function of these endoneurial neutrophils was unknown and never mentioned in literature regarding Wallerian degeneration. Occasional polymorphonuclear leukocytes enter injured or regenerating peripheral nerves[6], but they are very infrequent [7]. But why other authors did not see many neutrophils as we did? Two probabilities are explained here. Since the polymorphonuclear cells or neutrophils are indistinguishable by their morphology of nuclei, most authors will have difficult to identify them as in human specimen. The other possibility is the antibody for neutrophil is very sensitive to fixatives, especially the MCA967 for rats. Only the mildest fixative such as cold acetone will get consistent positive staining.

In a study by Anderson et al [8] of unilateral injection of the excitotoxin kainic acid into the mouse hippocampus. They did not observe any neutrophil recruitment but only enhanced expression of F4/80 on mononuclear phagocytes. The absence of neutrophil recruitment and the delay in an increase in macrophage or microglial cells shows that the CNS differs from other sites in the body with regard to the kinetics and nature of the myelomonocytic cell inflammatory response. But from the series studies in this thesis, we found that the peripheral nerve injury will elicit a spectrum of inflammation processes, even including the T cells[9] and neutrophils. Maybe the blood nerve barriers are not so tight in terms of the inflammatory cell extravasations than those of the blood-brain barriers. In conclusion, neutrophils are the initiator of inflammation including Wallerian degeneration

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