計畫名稱：鼠坐骨神經移植的血管新生：高壓氧的效果
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摘要：
周圍神經因外傷或腫瘤切除造成缺損時須用自體神經移植。移植後的神經軸突能否再生，與移植神經的血液循環是否迅速建立有關。本實驗的目的是比較未冷凍及冷凍過的自體神經移植，血管新生的情形；同時評估高壓氧是否促進血管新生。我們將一側一段2公分的大鼠坐骨神經取下，經五次冷凍解凍後，以顯微手術接回;另一組的神經移植則不經冷凍處理，在手術後2d, 4d, 7d, 及14d 將檢體分為各一公分的四組: 1)近吻合區2) 移植神經中段3)遠吻合區4)對側對照組。以細胞免疫方法用anti-endothelial cell (RECA-1)抗體染色所有血管，以定量新生血管。我們再以細胞免疫方法定位VEGF由何種細胞產生。The VEGF positive cells are found to be round macrophge-like cells around the perineurium of nerve graft. Frozen-nerve graft induced more angiogenesis as revealed by RECA-1 antibody.

關鍵詞: (英文)
Nerve graft; endothelial cell growth factors; peripheral nerves; nerve regeneration; hyperbaric oxyten

一、計畫簡介（Introduction）

Surgical reconstruction of extensive peripheral nerve injuries frequently require autogenous nerve grafts. Nerve grafts would offer a conduit for nerve regeneration into distal targets. The graft will undergo Wallerian degeneration in addition to the processes of revascularization. The invading monocytes/macrophages in the degenerating nerves extravasate and migrate from the proximal and distal junction area in a model of freeze in situ injury in rats. (Unpublished result, H F Chien and T Y Shieh) The vessels in the junction between injured and intact zones must undergo angiogenesis to support the devascularized nerve and the cells within. We are interested in the degree of angiogenesis and what endothelial growth factors involved. Although there are lots of reports on tumor or tissue angiogenesis, there is no report on this aspect of nerve regeneration so far.

Angiogenic factors were found to be important in wound healing.(Hom and Maisel, 1992). Recently, vascular endothelial growth factor (VEGF; also known as vascular permeability factor) was found to be important in neovascularization also in increased permeability which was the fundamental disease mechanism such as in diabetic retinopathy.(Dvorak et al., 1995) VEGF probably functions as a

hypoxia-inducible angiogenic factor. (Shweiki et al., 1992) VEGF induces the proliferation of endothelial cells and is a potent angiogenic factor that binds to heparin. Cell surface-associated heparin-like molecules are required for the interaction of VEGF with its cell surface receptors.(Gitay-Goren et al., 1992)VEGF mRNA undergoes alternative splicing events that generate four different homodimeric isoforms, VEGF121, VEGF165, VEGF189, or VEGF206. VEGF121 is a nonheparin-binding acidic protein, which is freely diffusible. The longer forms, VEGF189 or VEGF206, are highly basic proteins tightly bound to extracellular heparin-containing proteoglycans.

There is no report on the VEGF expression in the nerve graft so far. This studies in the first year will provide baseline information about angiogenesis phenomenon in nerve grafts, either cellular or acellular. Besides, the effect of HBO on nerve angiogenesis was studied.

二、材料及方法(Subjects and Methods)

Wistar rats ranging 250-300 g are used. Before operation animals are given pentobarbital (45 mg/kg) intraperitoneally and prophylactic antibiotic of cephalosporin intravenously. Nerve grafts will be prepared either fresh or frozen. For frozen grafts, a 2-cm segment of right sciatic nerve is removed and froze (-70°C) and thawed repeatedly 5 times with puffs of Chlorodifluoromethane (Freeze-it E, Shandon, U.K.). In this manner, all the cells are killed.(Ide and Kato, 1990)Under microscope, the nerve grafts are isotopically anastomosed with 2 sutures of 10-0 Nylon on each proximal and distal anastomosis. For fresh grafts, the nerve grafts are taken and reanastomosed without freezing. At 2d, 4d, 7d and 14d, 3 Wistar rats each groups are sacrificed under deep anaesthesia.

Hyperbaric oxygen therapy of 2.1 atm for 1.5 hours was performed for 10 days after reanastomosis and the vascular density by RECA-1 immunostaining was evaluated on day 14.

For light microscopy (LM), the animals were perfused with warm Ringer's solution followed by an aldehyde fixative composed of a mixture of periodate-lysine-paraformaldehyde, containing 2% paraformaldehyde, according to the method of McLean and Nakane.(McLean and Nakane, 1974) Specimens of cryoprotected sciatic nerve were placed in O.C.T. embedding material and snap frozen in melting isopentane cooled with liquid nitrogen. Cryostat sections of 10 μm thick were mounted on silanized slides and ready for use or stored at -70°C for future use.

Before immunostaining with monoclonal antibodies, endogenous peroxidase activity was abrogated by incubation of the sections in 3% hydrogen peroxide for 30 min. The sections were washed with TBS. The sections were incubated overnight at 4°C with the following antibodies: (1) RECA-1 antibody (1:100, Boehringer

Mannheim) 2) VEGF monoclonal antibody against $VEGF_{121}$ (Upstate, NY, USA)(Zhang et al., 1997) Immunostaining was performed with ABC method and DAB as peroxidase substrate. Vaculature density was evaluated. By applying an unbiased counting frame of known area (6400 micrometer^2)(Gundersen, 1977) systematic non-overlapping series of fields were examined across the whole nerve section at a constant step size. The number and area of vessel stained with anti-endothelial antibody (RECA-1) are calculated with the aid of image analysis program.

三、結果(Results)

The vasculature density of normal nerve tissue was demonstrated in Fig 1A. The brownish staining was much increased in frozen nerve graft at 7d. (Fig 1B) We also detected that hyperbaric oxygen treatment increases vascuature density significantly. (Data not shown) The vasculature as revealed by RECA-1 antibody also increased by 14d. The frozen nerve grafts contained more vascuature as compared with fresh non-frozen ones.

The VEGF immunostaining was first detected at 2d around the perineurium at suture site within cells of leukocytes-like. (Fig 2A) The number and intensity of VEGF positive cells increased at 4d (Fig 2B), 7d (Fig 2C, D, E) and 14d (Fig 2F). There is some staining around the degenerating myelin. (arrowheads in Fig 2B) The significance of this staining need further confirmation. By double labeling with VEGF and S-100 (polyclonal antibody for Schwann cells, Sigma, USA) (dark blue-color SG reaction product, arrowheads in Fig 2D and 2E) showed that most of the VEGF postive cells are around the perineurium and not to be Schwann cells. By 14d, there were still abundant VEGF positive cells.

四、討論(Discussion)

VEGF was found to be produced by adipocytes (Zhang et al., 1997)or neurons and axons(Samii et al., 1999) We now first demonstrated it within macrophages. Although we need a double labeling of VEGF with macrophage marker, we can tell the cell type by their nuclear shapes. But in situ hybridization for VEGF mRNA is still pending.

The primers and probe of VEGF121 cDNA were designed and prepared recently. I am now performing the realtime PCR quantification now. In situ hybridization for mRNA of VEGF will soon be done also.

Conclusion: the VEGF positive cells are found to be round macrophge-likes cell around the perineurium of nerve graft. Frozen-nerve graft induced more angiogenesis. Hyperbaric oxygen therapy could increase neo-angiogenesis in frozen and non-frozen grafts.

五、參考文獻(References)

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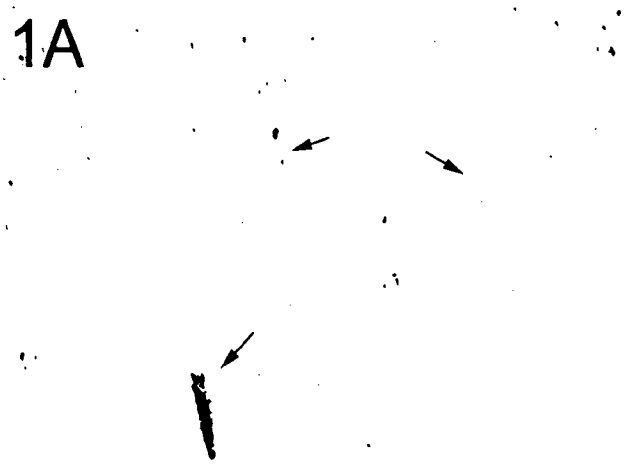
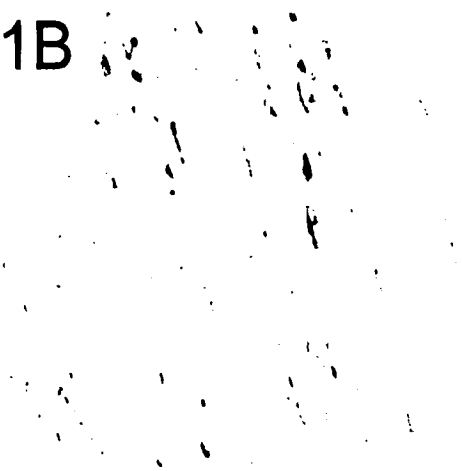
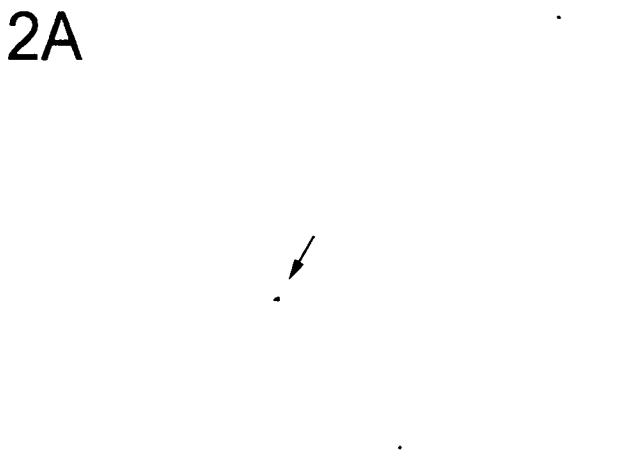
1A



1B



2A



2B

2C

2D

2E

2F