# Methylcobalamin promotes angiogenesis after peripheral nerve injury.

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INTRODUCTION: The importance of angiogenesis in an early stage after peripheral nerve injury and the neuroregenerative effects of methylcobalamin (MeCbl) are already known, however, the effects of MeCbl on angiogenesis remains unclear. The purpose of this study is to investigate the effects of MeCbl on angiogenesis in a rat peripheral nerve injury model.

METHODS: For *in vivo* experiments, we used 18 male Wistar rats (6 weeks old, 200g). The left sciatic nerve with 5mm length was excised, which was decellularized by frozen and thaw cycle for five times using liquid nitrogen. The nerve defect was bridged using the decellularized nerve tissue. Then, an osmotic pump adjusted to release MeCbl at a concentration of 1mg/kg/day was placed subcutaneously (MeCbl group), while the other group received an osmotic pump containing saline (control group). At postoperative 3, 5, and 7 days, nerves were harvested and evaluated for immunohistochemistry. For *in vitro* experiments, cell proliferation (BrdU assay), migration (scratch assay), and angiogenesis (tube formation assay) were evaluated in the presence or absence of MeCbl using Human Umbilical Vein Endothelial Cells (HUVECs). Additionally, we performed receptor tyrosine kinase (RTK) assay, RAS protein detective assay and Western Blotting to assess intracellular signaling pathways influenced by MeCbl. Statistical analysis was performed using unpaired Student's *t*-test or one-way ANOVA with Tukey – Kramer's multiple comparison test when experiments contained more than two groups. The Dunnet multiple comparison test was performed when appropriate.

RESULTS SECTION: In the immunohistochemistry for sciatic nerves, the neovascular areas, labeling for nestin (red), significantly increased in the MeCbl group at postoperative day 3 and 5 ($p<0.01$) compared with the control group. From the perspective of the bridging by Schwann cells, MeCbl group showed a significant increase of Schwann cell positive area at postoperative day 7 ($p<0.05$). The scratch assay and the tube formation assay indicated the significant increase for cell migration and angiogenesis in the MeCbl group ($p<0.01$), whereas there was no significant difference between each group in the BrdU assay. Furthermore, in the RTK assay, MeCbl was found to have no effects on the activation of receptor tyrosine kinases (RTKs) on the cell membrane surface. However, through the RAS protein assay, it was elucidated that MeCbl activates RAS proteins, downstream of the RTKs. Moreover, Western Blotting showed that MeCbl activated the PI3K – AKT – mTOR pathways, which is a downstream signaling cascade of RAS, more than control group. By inhibiting the effects of MeCbl, these activities significantly decreased, becoming equivalent to the Control group.

DISCUSSION: Our study revealed that MeCbl promotes angiogenesis in the early stages of peripheral nerve injury *in vivo*. Considering the *in vitro* results, the mechanism underlying this effect is thought to be different from growth factors like VEGF, which interact with receptor tyrosine kinases (RTKs) on the cell membrane surface. Instead, MeCbl is considered to activate the expression of RAS proteins not through these receptors. Recent research has reported that through the methylation cycle, MeCbl accelerates the convert of methionine into S-Adenocyl Methionine (SAM). Another research has also reported, SAM is utilized by isoprenylcysteine carboxyl methyltransferase (ICMT), resulting in the conversion of inactive RAS proteins to its active form. Based on these facts, it is hypothesized that MeCbl leads to an elevation of SAM, which, in turn, activates RAS proteins by methylation, thereby triggering the activation of downstream signaling pathways and promoting angiogenesis. In the present experiment, MeCbl promoted the migration and tube formation of HUVECs but did not promote proliferation. These results are consistent with the result that the ERK pathway, which is involved in cell proliferation, was not activated. However, the ERK pathway is also originally present in the downstream of the RAS protein, which is inconsistent with the present result that activation of the RAS protein did not activate the ERK pathway. There are some reports that RAS proteins exist in various subtypes, among which a subtype is mainly responsible for activating the PI3K-AKT-mTOR pathway. Hence, the possibility arises that MeCbl may selectively target specific RAS subtypes and express their effects. Further studies are needed to identify these subtypes and to elucidate the detailed mechanisms of the effect of MeCbl.

SIGNIFICANCE/CLINICAL RELEVANCE: Our study revealed that MeCbl promotes angiogenesis following peripheral nerve injury in a rat model. Our results may lead to an application of MeCbl not only for nerve regeneration but also for the regeneration of other tissues.

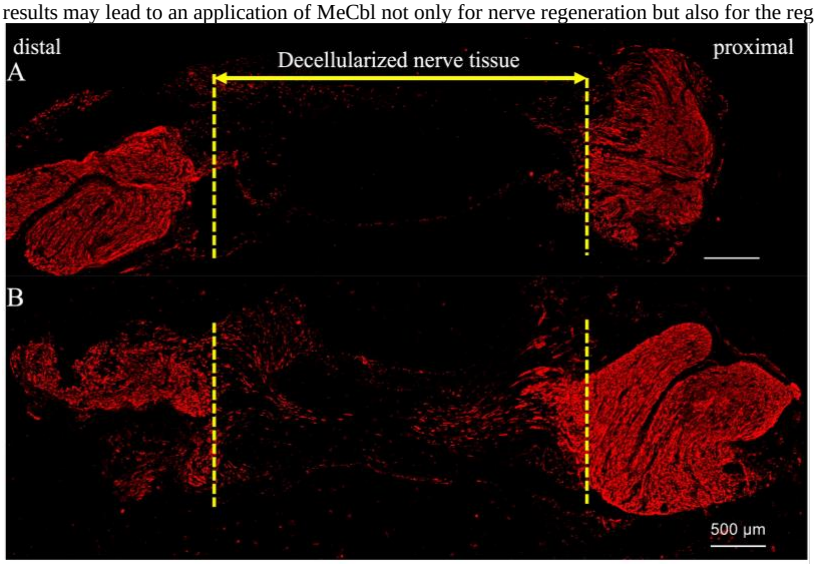


Figure 1 : Immunohistochemistry images for sciatic nerves at postoperative day 3. These images represent for neovascular areas with labeling for nestin (red) . A : control group. B : MeCbl group; scale bars =500μm.

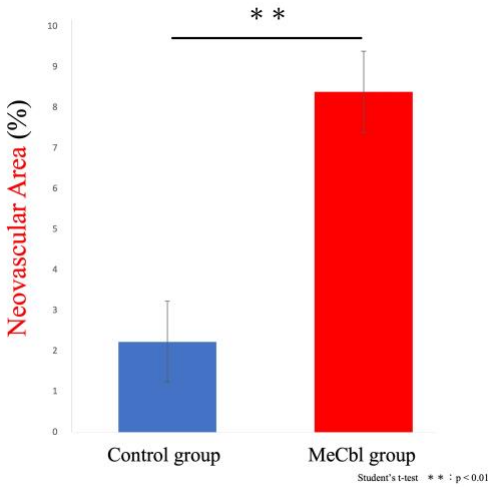


Figure 2 : Neovascular area was assessed by Image J.
The neovascular areas significantly increased in the MeCbl group at postoperative day 3