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Effects of erythropoietin on glial scar formation and axonal outgrowth in a model of glial scarring analogous to traumatically- injured spinal cord

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Objects: To develop an ideal in vitro model analogous to environments of traumatically-injured spinal cord using kainic acid (KA) and investigate the effectiveness, mechanism, and therapeutic time window of erythropoietin (EPO) in attenuating the glial scar formation.

Materials and Methods: Astrocyte cultures from spinal cord were obtained from postnatal day 6 Sprague-Dawley rat pups. After removal of loosely adhering oligodendroglia and strongly adhering microglia by shaking or using mM L-leucine-methylester, cells were allowed to grow to near confluence in DMEM containing 20 mM glucose and 10% calf serum in a water saturated air with 5% CO^2 at 37°C. Following culture > 3 weeks, 99% of the cells were GFAP (glial fibrillary acidic protein)-positive astrocytes were confirmed using immunocytochemical staining. To develop in vitro model, the cells were treated with KA at different concentrations (10, 50 or 100 μM) for 2 hours for chemical injury. For mechanical injury (S), uniform scratch wounds were made using a standard 200 μl sterile plastic pipette tip by removing strip of cells; two horizontal and two vertical lines were drawn for 'moderate' scratch model and three horizontal and three vertical lines for 'extensive'. For mixed injury (S/K), 'extensive' scratch wound and followed treatment with KA were provided. For investigation of EPO, extensive scratch wound and treatment with KA of 50 μM for 2 hours were provided. After those, EPO treatment at different concentrations (0, 100, or 300 U/ml) was provided 0, 2, 4, 8 hours and cultured for 48 hours. For evaluation of the neurite extension, spinal cord neurons from E-16 Sprague-Dawley rat embryos were plated onto the astrocyte cultures at the density 2×10^5 cells immediately after S/K injury and were treated with EPO (100 U/ml) at 0, 2, 4, 8 and 12 hours after S/K injury. Some cultures were treated with KA inhibitor or anti-rhEPO receptor antibody. Confocal microscopic exam and immune-histochemical analyses were performed.

Results: The optical immune-densities of GFAP, vimentin, CSPG, ROCK, or EphA4 were intensified in proportion to concentrations of KA ($p<0.001$) and was more prominent in extensive scratch injury than moderate one ($p<0.001$). The optical immune-densities of GFAP, vimentin, CSPG, and ROCK in S/K were the most prominent among control, KA, S, or S/K model ($p<0.001$). The immuno-positive area fraction of GFAP and phosphacan was the biggest and that of β-III tubulin the smallest in S/K among control, S, S/K, and KA inhibitor treated S/K ($p<0.001$). The immuno-positive area fraction of β-III tubulin in kainate inhibitor treated S/K model was more increased than that in S/K ($p<0.001$). EPO treatment 0, 2, 4 hours reduced the expression of GFAP, vimentin, CSPG, phosphacan, ROCK, EphA4, TNF-α, TFG-β, and p-Smad3 ($p<0.001$) and promoted β-III tubulin-immunoreactive axons ($p<0.001$). EPO-enhanced β-III tubulin-immunoreactive axons was inhibited by anti-rhEPO receptor antibody ($p<0.001$).

Conclusion: KA co-treatments in addition to a scratch induced glial scar and inhibiting molecules and restricted neurite outgrowth more strongly than either one. The current in-vitro model may be more useful tools for researching the therapeutic strategy for traumatically-injured spinal cord. EPO treatment within 4 hours after injury reduced astrogliosis, promoted neuritis outgrowth, and inhibited the transcription of pro-inflammatory cytokine. EPO treatment within the appropriate therapeutic time window may be useful tools for regeneration after spinal cord injury.

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