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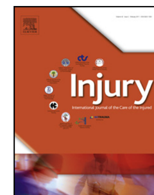

Epidermal growth factor regulates apoptosis and oxidative stress in a rat model of spinal cord injury

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ABSTRACT

Spinal cord injury (SCI) leads to vascular damage and disruption of blood-spinal cord barrier which participates in secondary nerve injury. Epidermal growth factor (EGF) is an endogenous protein which regulates cell proliferation, growth and differention. Previous studies reported that EGF exerts neuroprotective effect in spinal cord after SCI. However, the molecular mechanisms underlying EGF-mediated protection in different regions of nervous system have not shown yet. In this study, we aimed to examine possible anti-apoptotic and protective roles of EGF not only in spinal cord but also in brain following SCI.

Twenty-eight adult rats were divided into four groups of seven animals each as follows: sham, trauma (SCI), SCI + EGF and SCI + methylprednisolone (MP) groups. The functional neurological deficits due to the SCI were assessed by behavioral analysis using the Basso, Beattie and Bresnahan (BBB) open-field locomotor test. The alterations in pro-/anti-apoptotic protein levels and antioxidant enzyme activities were measured in spinal cord and frontal cortex.

In our study, EGF promoted locomotor recovery and motor neuron survival of SCI rats. EGF treatment significantly decreased Bax and increased Bcl-2 protein expressions both in spinal cord and brain when compared to SCI group. Moreover, antioxidant enzyme activities including catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) were increased following EGF treatment similar to MP treatment. Our experiment also suggests that alteration of the ratio of Bcl-2 to Bax may result from decreased apoptosis following EGF treatment. As a conclusion, these results show, for the first time, that administration of EGF exerts its protection via regulating apoptotic and oxidative pathways in response to spinal cord injury in different regions of central nervous system.

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Introduction

Central nervous system cells are highly vulnerable to various insults due to its limited regeneration capacity. Moreover, primary and secondary injuries including free radicals and ischemia-reperfusion injury lead to defects on axonal regeneration and functional recovery. Since the spinal cord is the connecting pathway for most neural control mechanisms, any damage in spinal cord can cause dysfunction in almost all biological systems. Neuronal damage occurring following spinal cord trauma results in inflammation, oxidative stress, motor neuron apoptosis, necrosis and autophagy [1]. Free radicals can react with and damage virtually any biological molecule, including DNA, proteins and membrane lipids. Antioxidant defence mechanisms such as GSH, catalase, superoxide dismutase (SOD), glutathione peroxidase (GPx) and vitamin E normally prevent or limit ROS production and tissue damage. It is well known that the enhanced production of reactive oxygen species (ROS) during spinal cord injury (SCI) appears to play an important role in neuronal cell death and neurological dysfunction [2].

Neuronal cell death occurred in spinal cord injury generally includes apoptosis, necrosis and autophagy. Since the characteristics of these cell death types differ, the mechanisms involved in

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regulation of cell death depend on the presence of proteins or enzymes in tissues. Although it is well known that apoptosis is a physiological process that occurs in cells during development and normal cellular processes, damaged cells die during the apoptotic process. Apoptosis is characterized by cellular shrinkage, plasma membrane blebbing, chromatine condensation, appearance of apoptotic body and nuclear degradation. As a marker of apoptosis, proteins responsible for regulation of intrinsic, extrintic cascades and DNA fragmentation are commonly used in experimental models. The activation of a family of intracellular cysteine proteases, caspases, plays important role in the initiation of apoptosis and activation of apoptotic processes [3]. The activated caspases including caspase-3 cleave poly (ADP) ribose polymerase, an enzyme which is involved in DNA damage repair and used as an early marker of apoptosis. Besides, some proteins including Bcl-2 family members are critically important for the regulation of apoptosis at the mitochondria. The translocation of pro-apoptotic Bcl-2 family protein Bax from the cytosol to the mitochondrial outer membrane results in opening pore in mitochondrial membrane and releasing of cytochrome c to the cytosol [4]. On the other hand, anti-apoptotic Bcl-2 protein blocks programmed cell death without affecting cellular proliferation [5]. The ratio of Bax to Bcl-2 determines the susceptibility of a cell to apoptosis [6,7]. It has been reported that microenvironmental depletion or functional inability of growth factors whose stimulus is necessary for cell survival, such as insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), fibroblast growth factor (FGF) is sufficient to induce neural cell apoptosis [8]. Thus, the systematic clinical utilization of different growth factors, alone or in combination, is among new strategies in the prevention and treatment of neurodegenerative disorders.

The environment of injured tissue restricts the regenerative capacity of endogenous neural stem/progenitor cells. Any strategies for overcoming such restrictions remain to be developed. In vivo infusions of exogenous growth factors into the fourth ventricle of the adult mouse brain is shown to increase the proliferation of neural progenitors around the fourth ventricle and the central canal of the spinal cord [9]. EGF a key molecule for remyelination, has been shown to stimulate the proliferation and differentiation of ependymal cells, a potential source of endogenous progenitor/ stem cells in the adult spinal cord [10]. Moreover, EGF has been shown to exert a neuroprotective effect [11] and provide potential therapeutic interventions for preventing blood-spinal cord barrier disruption after SCI [12]. On the other hand, previous studies reported that the inhibition of EGF receptor attenuates reactive astrogliosis and tissue damage, improves functional outcome in animal models [13,14].

There are still limited and controversial studies about the therapeutic potential of EGF. Thus, the present study, focused on spinal cord and frontal cortex, was designed to investigate the changes in locomotor recovery, antioxidant capacity (catalase, SOD, GPx), and apoptosis (Bax, Bcl-2, caspase-3, cleaved caspase-3) after EGF treatment following SCI in a region-specific manner.

Materials and methods

The animals were handled with care under the prescriptions for animal care and experimentation of the relevant European Communities Council Directive (86/609/EEC) and all the procedures were approved by the Institutional Animal Ethics Committee of Ege University, Izmir, Turkey (Institutional Animal Care and Use Committee approval No. 2015-010). Adult Sprague–Dawley rats (200–300 g) were obtained from the Kobay A.S. (Ankara, 220615-260-SD). All efforts were made to minimize the number of animals used during the procedure and their suffering. The maintenance and operation of the rats were carried out in Ege University ARGEFAR Experimental Animal Laboratory. All animals were maintained on a 12:12 h darklight cycle, with free access to chow and water. The temperature level in the chamber was kept at 20–24 °C and the humidity level was between 50 and 60%. Lyophilized recombinant human EGF (Heberprot-P®, Center for Genetic Engineering and Biotechnology (CIGB), Havana, Cuba) containing 75 μg of rhEGF per vial, was dissolved with 5 ml of water for injection.

Animals and SCI

Adult male Sprague–Dawley rats (n = 28) were divided into four groups of seven animals each: (i) control (Sham), (ii) Trauma (SCI), (iii) Trauma (SCI) + EGF, (iv) Trauma (SCI) + Methylprednisolone (MP). Intraperitoneal anesthesia was performed with ketamine hydrochloride (50 mg/kg; Ketalar, Parke-Devis, Istanbul, Turkey) and xylazine hydrochloride (10 mg/kg; Rompun, Bayer, Istanbul, Turkey) while anaesthetizing them. The surgical incision was made at the back of the rat, after shaving and obtaining the aseptic conditions. Rivlin and Tator's clip compression model was applied in the study [15,16]. The skin, subcutaneous tissue, and paravertebral muscle fascia from the level of T6-T11 were passed in the prone position based on the interscapular distance, and the muscles were scraped by a lateral blunt dissection. Bilaterally laminectomy was performed between the T8-T10 segments. When this procedure was carried out, care was taken not to damage the subjects' dura maters. Spinal cord compression trauma was then created by applying the Rivlin and Tator clip compression method for one minute to the animals. Sham groups were only subjected to laminectomy. In order to standardize trauma, Yaşargil Aesculap® FE 654 type aneurysm clip with extradural closure pressure of 30 g was used.

Drug treatment

MP-treated animals were used to compare the efficacy of EGF against oxidative stress and cell death mechanisms [17,18]. MP was administered intraperitoneally (30 mg/kg) within 1 h after the surgical incision was closed according to the National Acute Spinal Cord Injury Study II protocol. As a maintenance dose, 124.2 mg/kg MP (5.4 mg/kg/h × 23 h) was injected subcutaneously through the interscapular area [19]. EGF (15 μg/ml, bolus,1 ml) was injected entering ~1 mm into dura locally into the trauma area with a sterilized micropipette (tip diameter <50 μm) attached to Hamilton syringe [20]. The rats were housed for a survival period of 8 days. Rats taken in separate cages were provided with urinary bladder massage manually three times a day in order to provide urine output.

Behavioural tests

The BBB locomotor rating scale was used to asses locomotor performances of the rats after SCI. The functional recovery of all rats at 0, 1, 3, 7, and 8 d after SCI was scored by two experimenters in an open-field through BBB scale [21]. At the end of the acute and sub-acute period (on the eighth postoperative day) all rats were sacrificed with high-dose anesthetic. Brain and spinal cord samples were dissected. Samples were stored at −80 °C until use.

Homogenization of tissues

The frozen samples were weighed and homogenized (1:10, w/v) in 20 mM phosphate buffer (pH 7.4) containing 140 mM potassium chloride at +4 °C. The homogenate was centrifuged at 1,200 × g for 10 min at +4 °C. The supernatant was used for antioxidant enzyme

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