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The effects of G-CSF and naproxen sodium on the serum TGF-β1 level and fracture healing in rat tibias

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Abstract

Local and systemic release of transforming growth factor beta 1 (TGF-β1) is known to increase during the process of fracture healing and this cytokine stimulates bone healing. The majority of the non steroidal anti inflammatory drugs (NSAIDs) inhibit fracture healing. Granulocyte colony stimulating factor (G-CSF) is a hematopoietic growth factor that stimulates bone marrow. In this study, the effects of the NSAID naproxen sodium, G-CSF, and both of them in combination on the TGF-β1 serum level in rats with tibia fractures were measured and fracture healing was evaluated by histopathologic and radiologic examination. The TGF-β1 serum levels obtained on day one (24 h after fracture but before administration of naproxen or G-CSF) were found to be similar in all of the five groups ($p>0.05$). At the end of the first week, TGF-β1 levels were significantly lower in naproxen-treated rats than those of the other groups excluding control ($p=0.002$). Similar changes in TGF-β1 levels were found at the end of the second and fourth weeks. TGF-β1 levels were significantly higher in G-CSF-treated rats at the end of the first, second and fourth weeks ($p<0.05$). Fracture healing scores measured with histopathological and radiological methods were higher in G-CSF-treated rats than in naproxen-treated ones. When both naproxen and G-CSF were given, the scores resumed to normal. The results point to the negative effect of naproxen sodium on fracture healing is due to its decreasing effect on the level of TGF-β1, which may be a new possible mechanism. Moreover, this negative effect can be inhibited by the use of G-CSF.

Keywords: Fracture healing; TGF-β1; G-CSF; Naproxen

Introduction

Fracture healing starts with a hematoma, continues with bone formation, and ends with bone remodeling. During these processes growth factors, especially transforming growth factor beta 1 (TGF-β), stimulate bone healing and formation (Dimitrou et al., 2005). The fact that there is a relationship between osteogenesis and hematogenesis has been put forward by Vaughan. It is known that in osteopetrosis both hematologic and osteologic pathology improve after the transfer of appropriate bone marrow (Vaughan, 1981). The granulocyte colony-stimulating factor (G-CSF) is a hematopoietic growth factor that enhances the proliferation and differentiation of neutrophil progenitor cells. G-CSF leads to an increase in bone marrow progenitor cells. Particularly, it prolongs the survival time of neutrophils, retarding the apoptosis of neutrophil (Adachi et al., 2003; Demetri and Griffin, 1991; Inano et al., 1998; Cohen et al., 1987; Ulich et al., 1988; Pojda and Tsuboi, 1990; Corti et al., 2002). G-CSF has been used clinically in the treatment of bone marrow transplantations and secondary granulocytopenia after chemotherapy (Schmitz et al., 1996; Adachi et al., 2003).

TGF-β1 however is a growth factor that has been described as five isoforms in mammals (Kingsley, 1994). Its most abundant form, TGF-β1, is found in platelets and leucocytes, and is

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released into the fracture hematoma (Dimitrou et al., 2005). After fracture its level increases both locally and systemically. An increase of released TGF-β1 has been observed during the healing process after femur fractures. It was reported that injections of TGF-β1 stimulated new mineralized bone formation in mice. TGF-β1 given systemically enhanced cancellous bone formation. On the other hand, it was shown that TGF-β enhanced collagen synthesis and particularly stimulated alkaline phosphatase activity in cell culture (Ibbotson et al., 1989; Joyce et al., 1990; Linkhart et al., 1996; Natsu-ume et al., 1997; Rosier et al., 1998; Lieberman et al., 2002; Schmidmaier et al., 2002; Yeung et al., 2002; Zhang et al., 2003). It has been found that endogenous release of TGF-β reached its peak level within a week following the injury (Natsu-ume et al., 1997; Dimitrou et al., 2005). Sciore et al. (1998) showed that TGF-β messenger RNA sustained a high level during first 3 weeks following the trauma. Regarding its relationship with G-CSF it is worth looking at Hirayama's investigation, which concluded that the use of G-CSF increased the level of TGF-β1 (Hirayama et al., 2002).

Naproxen is a widely-used non steroidal anti-inflammatory drug (NSAID). Most of the NSAIDs have a negative effect on fracture healing, spinal fusion, and bone in-growth into porous structures of prostheses (Allen et al., 1980; Keller et al., 1989; Trancik et al., 1989; Hogevold et al., 1992; Cook et al., 1995; Dimar et al., 1996; Giannoudis et al., 2000; Simon et al., 2002). In an experiment about the interaction of NSAIDs and cytokines, it was found that cyclooxygenase 2 (COX-2) inhibitors had a decreasing effect on the level of interleukin-6 and its mRNA (Anderson et al., 1996). Although there is a lot of scientific evidence showing that NSAIDs have a negative effect on fracture healing, to the authors' knowledge the mutual interaction between NSAIDs on the one hand and TGF-β1 and G-CSF on the other hand during fracture healing has not been investigated specifically so far. For this reason we decided to investigate the effect of naproxen sodium, G-CSF and both of them in combined mode on the serum level of TGF-β1 during fracture healing. The healing was also evaluated in microscopic and radiological examinations.

Materials and methods

Animals

A total of 35 male Wistar rats (mean weight of 300 g), aged 8 weeks obtained from the University of Erciyes Hakan Cetinsaya Laboratories (Kayseri, Turkey) were maintained in a controlled condition of 12 h light/12 h dark at 23±1 °C temperature and humidity 55±10%. Animals were fed a regular pellet diet and tap water. They were housed in specific cages and had free access to food. Housing, animal environment and all experimental procedures conformed to the American Council for Animal Care guidelines and were approved by Institutional Animal Care and Use Committee at the University of Inonu.

Tibia fracture model

Standardized closed tibia and fibula fractures were inflicted manually on the middle of the bone as previously described by An and Friedman (1999). Under sterile conditions using general anesthesia with ketamine (60 mg/kg) and xylazine (10 mg/kg), tibia fractures were fixed with intramedullar fixation using 21G sterile needles. Full weight bearing was allowed immediately. The animals were divided randomly in the following five groups: Control group($n=7$): no treatment and no fracture; second group ($n=7$): fracture was inflicted; third group ($n=7$): fracture was inflicted and naproxen sodium (AÝ drug Company, Turkey 5.5 mg/kg/daily) was given to the rat throughout the experiment; fourth group ($n=7$): fracture was inflicted and G-CSF (Neupogen, Hoffman La Roche, Switzerland 10 μg/kg, 500 μl/7 days s.c.) was used; fifth group ($n=7$): fracture was inflicted and both naproxen sodium and G-CSF were used. The administration of G-CSF and naproxen sodium started on day 1 after the operation. These doses are in the range of the average recommended therapeutic doses for humans. Naproxen sodium was mixed with chocolate and fed to each rat daily to ensure consistent and accurate dosing (Long et al., 2002). In order to ensure sufficient dosing, each rat was taken out of its cage, fed a chocolate ball and then put back.

Histopathological examination

At the end of the 4th week all animals were killed with an injection of a lethal dose of pentobarbital. Each right tibia was disarticulated immediately from proximal to distal articulation. The surrounding soft tissue and intramedullar implant were carefully removed. Each specimen was placed in 10% normal buffered formaldehyde for 24 h at 4 °C and decalcified in 10% formic acid for 48 h at room temperature. Samples were embedded in paraffin. Using a microtome, 5-μm thick longitudinal sections were taken from the specimens. Sections were stained with hematoxylin and eosin (HE), and evaluated with light microscope. The grade of healing was determined according to a classification system of Emery (Lane and Sandhu, 1987). Microscopic analysis was performed by two pathologists who were blinded to the identity of the specimen.

Radiographic evaluation

Radiographs were taken in the antero-posterior and lateral view at the end of the 4th week. The bridging of fracture callus was described in randomly chosen animals from each group by two independent observers who were blinded to the identity of the specimens. Radiographic evaluation and grading were performed using the Lane and Sandhu scoring system (Lane and Sandhu, 1987).

Serum analyses

Blood samples (1 ml each) were taken by intracardiac puncture on day 1 and in weeks 1, 2 and 4, and stored at −20 °C until analysis (Bravenboer et al., 2001). Serum TGF-β1 concentration was measured with a commercially available enzyme-linked immunosorbent assay (ELISA) (BMS608/2 m TGF-β1, Bender Med Systems, CA, USA).