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The effect of bevacizumab and 5-Fluorouracil combination on epidural fibrosis in a rat laminectomy model

U. OZKAN, A. OSUN, A. SAMANCIOGLU, S. ERCAN[1], U. FIRAT[2], S. KEMALOGLU[1]

Department of Neurosurgery, Dumlupinar University Medical Faculty, Kütahya, Turkey
[1]Department of Neurosurgery, [2]Department of Pathology; Dicle University Medical Faculty, Diyarbakir, Turkey

Abstract. – OBJECTIVE: An animal model of laminectomy in rats was used to study scar tissue formation around the spinal cord. Bevacizumab (BV) [a recombinant, humanized, monoclonal antibody targeting vascular endothelial growth factor], 5-Fluorouracil (5-FU) and BV+5-FU was tested in this system for its ability to decrease fibrous tissue formation.

MATERIALS AND METHODS: Twenty-eight Sprague Dawley rats were used in this sudy. Rats were divided into four groups; a control group, a BV group, a 5-FU group and a BV+5-FU group. L1-2 laminectomies were performed on the rats. The medicated groups were treated with topical drug administration. After 6 weeks, the rats were sacrified and histologic sections prepared from the spines were examined and graded by a pathologist. Epidural fibrosis and fibroblast density were evaluated under light microscope.

RESULTS: BV (Avastin: Genentech, San Francisco, CA, USA) significantly reduced the density of the scar tissue undermining the laminas ($p < 0.005$). Monotherapy with 5-FU did not change the scar formation in the back ($p = 0.317$). Combination of 5-FU and BV was more effective on reducing the epidural fibrosis after laminectomy on rats ($p < 0.001$).

CONCLUSIONS: Bevacizumab reduced the spinal epidural fibrosis significantly that developed in rats after laminectomy and 5-Fluorouracil combination had a synergic effect. Further investigations under the light of these findings may help to reduce epidural fibrosis formation after laminectomy.

Key Words:
Laminectomy, Epidural fibrosis, Bevacizumab, 5-Fluorouracil.

Introduction

Postlaminectomy peridural fibrosis, which causes symptom recurrence due to compression or tethering of the nerve roots, is a well-known complication of lumbar disc surgery. Despite improvements of surgical techniques as well as adoption of microsurgical approach, the failed back syndrome represents with prevalence of 5-10% a major problem in spine surgery. The formation of postoperative epidural fibrosis with nerve root entrapment and dural compression is said to be the most common cause[1-4]. Since scar excision surgery generally yields poor results, many authors have suggested that prevention of postoperative adhesions is an essential goal in low back surgery.

Background

In the last years, a number of investigators have studied the effectiveness of various treatment for preventing epidural fibrosis with variable success. Some antineoplastic agents such as mitomycin C, 5-fluorouracil, cyclosporin and some high molecular weight molecules such as hyaluronan, oxidized regenerated cellulose, free fat grafts, steroid solutions, dural adhesion barriers, various chemical agents, and many surgical techniques such as smooth dissection or preservation of ligamentum flavum, and even external beam radiation therapy have been used to reduce or inhibit epidural fibrosis formation[5-14].

Angiogenesis is the formation of new blood vessels by remodeling and expansion of primary vessels; it is important in normal physiologic processes, including tissue growth, wound healing, fetal development, and reproductive function. Vascular endothelial growth factor (VEGF) is a potent, multifunctional cytokine that exerts several important and possibly independent actions on vascular endothelium. Increased vascular permeability has since been shown to occur during the early phases of wound repair, theoretically allowing deposition of the fibrin-rich matrix necessary

Corresponding Author: Arif Osun, MD; e-mail: arifosun@yahoo.com

for cellular migration and proliferation[15,16]. Bevacizumab (BV) (Avastin: Genentech, San Francisco, CA, USA) is a monoclonal antibody that binds and inactivates all isoforms of VEGF to inhibit angiogenesis and tumor growth and proliferation. It has been approved by the U.S. Food and Drug Administration (FDA) as a therapeutic agent for widespread metastatic colorectal cancer[4,17-19].

5-Fluorouracil (5-FU), a pyrimidine analog widely used in cancer chemotherapy and in glaucoma surgery, has recently shown some efficacy in the treatment of keloids, scars that overgrow the boundaries of original wounds[2,3,20]. Wendling et al[20] showed that FU prevents transforming growth factor-β (TGF-β) induced COL1A2 gene transactivation in human fibroblasts. Experimentally, 5-FU has been demonstrated to be a very effective inhibitor of fibroblast growth[2,3]. Khaw et al[21] showed *in vitro* and *in vivo* that a 5-minute exposure results in growth arrest and may have a long lasting effect on cultured human Tenon's fibroblasts. 5-FU also specifically interferes with qualitative fibroblast functions such as collagen lattice contraction[22]. It is particularly useful as an adjunct to inhibit wound healing.

Aim

In this study we examined the effects of BV, 5-FU and their combination on postoperative epidural fibrosis formation on rats.

Materials and Methods

28 Sprague Dawley rats (250-300 g) were housed in an air conditioned room with 12 h light and dark cycles, where the temperature (23±2°C) and relative humidity (65-70%) were kept constant. All experimental protocols were approved by Dicle University School of Medicine Animal Care and Use Committee (DUHADEK).

All rats had normal motor functions. Anesthesia was induced by intramuscular injections of ketamine (60 mg/kg) and xylazine (9 mg/kg). Animals were allowed to breathe spontaneously. The core temperature was monitored with rectal probe. The lumbar area of each rat was shaved, and the operative field was prepared in a sterile manner using povidone-iodine solution. A dorsal skin incision was made with a number-15 blade and continued down to the spinous process. The paraspinal muscles were stripped away from the lamina and spinous process. Under an operating microscope, laminectomies at L1 and L2 were performed with a 1 mm rongeur. The ligaments at L1-L2 were removed, and the underlying dura mater was exposed for 4 × 4 mm^2 widening. Throughout the procedure, haemostasis was maintained by irrigation with saline. Bipolar cautery, bone wax, surgical, or other hemostatic materials were not used. When the laminectomy site was free of active haemorrhage, it was irrigated with 10 ml of saline. To this point, the procedure was the same for all rats. Subsequently, the rats were assigned randomly to four groups of seven animals each. Group 1: (G1= control) laminectomy + no medication, Group 2 (G2): laminectomy + 5-FU, Group 3 (G3): laminectomy + BV, Group 4: (G4) laminectomy + BV + 5-FU. Cotton pads (4 × 4 mm^2) soaked with BV (2.5 mg/ml), 5-FU (5 mg/ml) and BV+5-FU mixture were applied on the exposed dura for 5 minutes. Then, the soaked cotton wool was removed and the laminectomy site was irrigated with 10 ml of saline to wash off any leftover agent. In the control group, the exposed dura mater was only irrigated with 10 ml of saline solution. In all groups, the facia at the laminectomy site was marked with 3/0 silk to facilitate the harvest of pathological specimens. The wound was then closed in layers using the same suture material in each animal. There were no complications or adverse effects from the surgery or the application of agents. No prophylactic antibiotics were used.

The rats were sacrified 6 weeks after surgery with a lethal dose (100 mg/kg) of sodium phenobarbital. The lumbar spine was removed en block whith the paraspinal musculature and fixed in 10% buffered formalin solution for 1 week and then placed in decalcifying solution until complete decalcification. The laminectomy site was identified and four 2 mm thick sections were obtained. Each section was embedded in paraffin and serial sections (5 μm) were cut with a microtome and stained with Haematoxylin & Eosin and Masson Trichrome for examination. All the sections were examined by a pathologist who was blinded to the groups. In the histopathological evaluation epidural fibrosis and fibroblast density were investigated.

Epidural fibrosis was graded based on the scheme devised by He et al[8]:

Grade 0: the dura is free of scar tissue;
Grade 1: only thin fibrous bands are observed between the scar tissue and dura;
Grade 2: continuous adherence is observed in less then two-thirds of the laminectomy defect;

Grade 3: scar tissue adherence is large, affecting more than two-thirds of the laminectomy defect, or the adherence extends to the nerve roots.

To quantify the fibroblast cells in the scar tissue, the cells in three different areas (two borders and the centre of the laminectomy defect) were counted and mean was calculated. The fibroblast densities were graded as follows:

Grade 1: less than 100 fibroblasts per × 400 field;
Grade 2: 100 to 150 fibroblasts per × 400 field;
Grade 3: more than 150 fibroblast cells per × 400 field.

Statistical Analysis

The Mann-Whitney U test was used to compare the data between groups. $p < 0.005$ was deemed statistically significant. The statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA).

Results

The postoperative course was uneventful in all animals. No appearance of wound infection could be detected, motor functions were normal, and there was no CSF leakage.

Epidural fibrosis formation evaluation revealed that there was no significant difference between the sham and 5-FU treated group (p = 0.317; Figures 1, 2). Comparing the BV medicated group with sham group showed that fibrosis formation was significantly decreased in BV group ($p < 0.005$; Figure 3). When we compared the 5-FU and BV medicated groups, BV was significantly effective than 5-FU in decreasing the epidural fibrosis formation ($p = 0.023$). Epidural fibrosis formation was least at BV+5-FU group ($p < 0.001$; Figure 4).

When we compared the same groups for fibroblastic activity, we found that there was no significant difference between the sham and 5-FU treated group as same as the result of epidural fibrosis formation ($p = 0.591$). Fibroblast density in the BV group was lower than the sham group ($p = 0.007$) and in the 5-FU + BV group was the least ($p = 0.004$). Comparing the

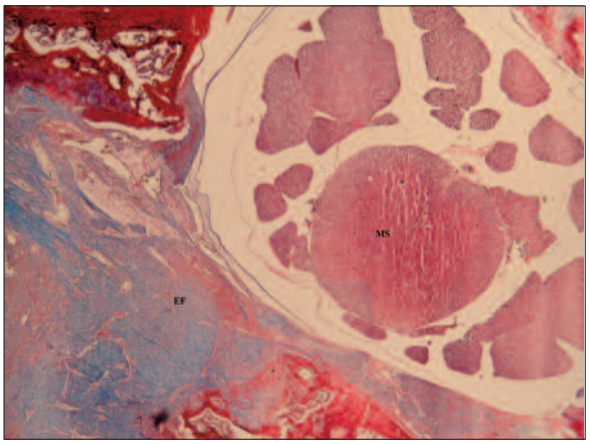

Figure 2. No significant difference between the sham and 5-FU medicated group. Masson Trichrome, × 40 (EF: Epidural Fibrosis, MS: Medulla Spinalis).

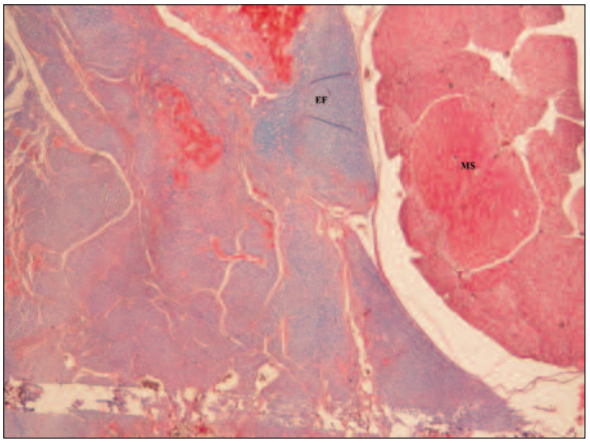

Figure 1. Thick epidural fibrosis formation in sham group. Masson Trichrome, × 40 (EF: Epidural Fibrosis, MS: Medulla Spinalis).

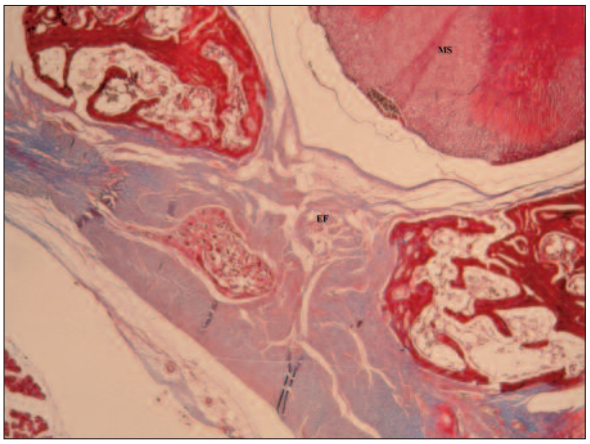

Figure 3. BV medicated group. Epidural fibrosis is significantly decreased. Masson Trichrome, X40 (EF: Epidural Fibrosis, MS: Medulla Spinalis).

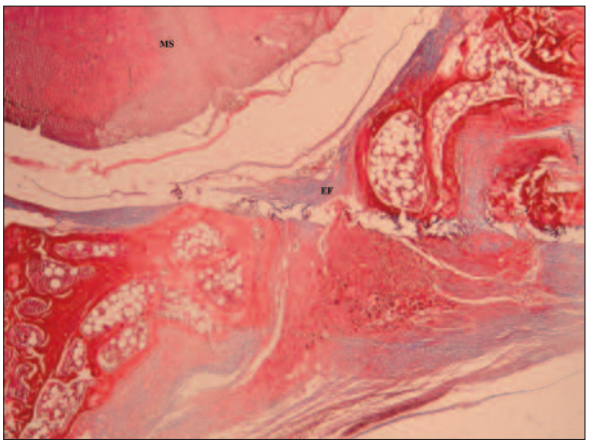

Figure 4. BV+5-FU medicated group. Epidural fibrosis is limited in a small area. Masson Trichrome, X40 (EF: Epidural Fibrosis, MS: Medulla Spinalis).

BV+5-FU group with the monotherapy groups revealed that fibroblastic activity was lower than 5-FU group ($p < 0.002$) and BV group ($p < 0.005$).

The results of epidural fibrosis and fibroblast density was shown as chart on Figure 5.

Discussion

Epidural fibrosis is one of the most common problems associated with spinal surgery. In this condition scar tissue adheres to the dura mater or nerve roots are formed in order to repair the local defect of the vertebral lamina created by the laminectomy. However, this scar formation may be compressive and restrict the mobility of the nerve root which often leads to an unfavourable clinical outcome. Concurrently epidural fibrosis increases the hazards of revision spine surgery and contributes to the occurrence of the "failed back surgery syndrome"[1]. Experimental studies have provided electrophysiological evidence of neurologic disturbances caused by peridural scar formation[23]. A multitude of other abnormalities including mechanical tethering of nerve roots secondary to epidural fibrosis in the vertebral canal[24], disturbances in blood flow[25], and expression of proinflammatory cytokines causing irritation of exposed dorsal root ganglion and triggering painful responses have been described[26].

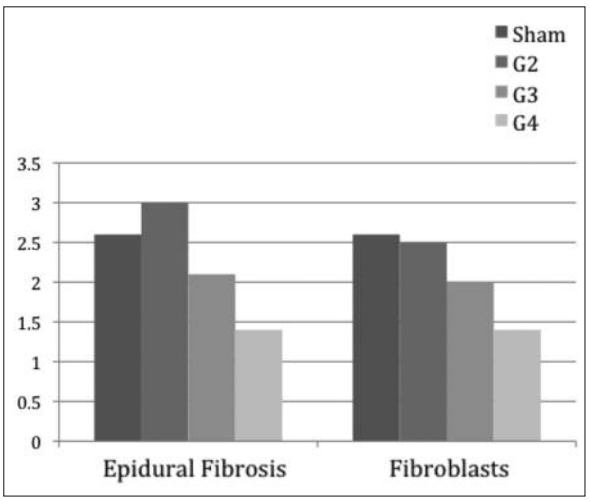

Figure 5. Epidural fibrosis and fibroblast density results of groups. Sham: Laminectomy without medication group; G2: 5-Fluorouracil group; G3: Bevacizumab group; G4: Bevacizumab+5-Fluorouracil group.

The physiopathology of epidural fibrosis is still controversial. The findings from animal research and clinical studies demonstrates that multiple factors (such as individual variability in degree of scar formation, postoperative haematoma, laminectomy technique, anatomic location within vertebral column, amount of bone removed) are involved in the pathogenesis of postoperative peridural scarring[7]. The peridural mechanism of peridural fibrosis is fibroblast migration into the surgical area and it seems to be a key factor affecting scar and peridural fibrosis formation[7]. Fibroblasts originated from perivertebral muscles and/or carried by blood into the operative area cause strong adhesion of the tissue.

Increased vascular permeability has since been shown to occur during the early phases of wound repair, theoretically allowing deposition of the fibrin-rich matrix necessary for cellular migration and proliferation[27]. An angiogenic response is also characteristic of the early phases of wound repair and provides the vasculature that supplies the newly formed granulation tissue. The discovery of increased VEGF protein production[28] and message expression[29] in skin wounds suggested that VEGF might regulate these two key wound events. Howdieshell et al[15] showed that VEGF neutralization produced a striking decrease in two wound environment parameters: wound fluid volume and angiogenesis. They also demonstrated that antibody neutralization of wound fluid VEGF produced significant reductions in granulation tissue thickness, wound fluid volume, vessel number, and vascular surface area. We used bevacizumab as a VEGF inhibitor and we found that epidural fibrosis formation was decreased and fibroblast density was reduced.

Many of the other prominent growth factors believed to have a role in wound repair, including platelet-derived growth factor (PDGF), the fibroblast growth factors (FGFs), and TGF-β have been functionally evaluated by their addition to the wound environment and found to promote small increases in granulation tissue mass or incisional wound breaking strength[30]. These are the parameters expected to be affected by increased fibroblast activity, such as collagen production and assembly, both of which are stimulated in fibroblasts in vitro by all three of these growth factors. Moreover, antibody neutralization of wound TGF-β reduced scarring in adult skin and stromal fibrosis in the injured cornea[31]. Howdieshell et al[15] showed progressive increase in wound fluid TGF-β 1 levels in laminectomy performed pigs.

Several different mechanisms could explain the excessive deposition of collagen in the fibrotic skin diseases. First, local expansion of synthetically active fibroblast populations, even with a normal rate of collagen production per cell, could lead to tissue accumulation of collagen. Second, the accumulation of collagen could result from accelerated production of collagen by fibroblasts, and such increase could reflect enhanced collagen gene expression at the transcriptional level[32]. Wendling et al[20] reported that 5-FU effects on type I collagen expression and subsequent collagen deposition could result from inhibition of TGF-β driven type I collagen transcription, and also they reported a molecular explanation for the observed clinical benefits of 5FU in the treatment of keloids.

In this study, 5-FU medication was not effective in decreasing the epidural fibrosis formation. We could see slightly decrease in fibroblast density at the laminectomy site. However, combination of 5-FU and BV had a striking effect on both epidural fibrosis formation and fibroblast density.

How et al[6] evaluated the effectiveness of combined treatment with BV and 5-fluorouracil to attenuate the postoperative scarring response after experimental glaucoma filtration surgery and they reported that the combined delivery of BV and 5-FU magnified the antifibrotic effect compared to the two agents separately.

Furthermore, it is important to note that BV and 5-FU are likely to be working synergistically to induce a more profound effect on fibrosis. It is proposed that the use of 5-FU together with BV would improve the postoperative wound healing response.

Conclusions

To date, anti-VEGF treatments have primarily targeted pathologic angiogenesis for both systemic and ocular neovascular disorders. As fibrosis is the most significant clinical problem that both hinders surgical success and is a principal cause of morbidity, our findings in this study suggest that adjunctive treatment with bevacizumab used in conjunction with 5-FU has the potential to further improve surgical outcomes.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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