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ORIGINAL ARTICLE

The Effect of Vascular Endothelial Growth Factor on Liver Regeneration in a Rat Model

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Abstract

Introduction: Stimulation of liver regeneration after hepatectomy may provide important advantages, especially in cases where liver tissue is at a critical level. Some studies have reported that vascular endothelial growth factor (VEGF) might ameliorate liver regeneration in animals. In this study, we aimed to examine the effects of exogenous VEGF164 on the regeneration of liver tissue after a 70% hepatectomy in rats.

Methods: Twenty Wistar Albino male rats, weighing 300-350 g, were randomly divided into four groups, each having five animals. Group 1 and Group 3 were designated as control groups, and Group 2 and Group 4 as experimental groups. All rats underwent laparotomy and 70% partial hepatectomy. Postoperatively, control groups were administered saline through the tail vein at 0 and 6 hours, and VEGF164 was administered to the experimental groups in the same manner at 0 and 6 hours. Blood samples were collected by sacrificing the rats at 24 hours for Group 1 and Group 2, and at 72 hours for Group 3 and Group 4. Subsequently, relaparotomy was performed and the remaining liver tissues were completely removed in all groups. Then, the resected liver tissues were used to determine morphological regeneration rates. The histopathological proliferation of the liver was evaluated by calculating the Ki-67 proliferative index.

Results: In the histopathological evaluation, it was revealed that the liver proliferation index was significantly higher in the VEGF164 group at 72 hours (p=0.028). Although liver weights were higher in the experimental groups, no statistically significant results were obtained in terms of morphological liver regeneration rates (p>0.05).

Discussion and Conclusion: Although the positive effects of exogenous VEGF on liver regeneration were not found in this rat model, statistically significant increases in the liver proliferation index are promising.

Keywords: Hepatectomy; proliferation; rat; regeneration; VEGF.

Liver resections are performed more frequently in recent years. Liver tissue remaining after major liver resections is closely associated with mortality and morbidity in the postoperative period.

Although the hepatocellular proliferation capacity of the liver is much better in healthy liver tissue rather than the tissue with parenchymal disease, the proliferation capacity in liver tissue with parenchymal disease is decreased

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in proportion to the degree of the disease[1,2]. In this setting, therapeutic strategies to increase regeneration and functional capacity of the remnant liver tissue are critical. Various studies have searched the effects of several agents on the regeneration capacity of the remnant liver tissue after resections[3-5]. Some published researches have revealed that vascular endothelial growth factor (VEGF) is upregulated after a 70% partial hepatectomy and that exogenous VEGF also improved proliferation of hepatocytes after partial resection[6,7]. However, there are still controversies on whether VEGF may increase liver regeneration after partial hepatectomy or not.

In experimental liver resections, the Higgins and Anderson model in which a 70% hepatectomy is made is frequently used[5]. In this study, we aimed to examine the effects of VEGF, which were administered to rats after a 70% hepatectomy performed using the same model, on liver regeneration.

Materials and Methods

Twenty Wistar Albino male rats, weighing 300-350 grams, were used in the study. One week before the study, the rats were taken to the research environment. Twelve-hour dark-light cycles were created for the rats to be acclimated. The rats were randomly divided into four groups, including five rats in each group. Group 1 and Group 3 were classified as control groups, and Group 2 and Group 4 as experimental groups. Each was placed in a single cage and fed with standard rat chow and tap water. Ethics Committee approval was received for the study (2009-50-248). ARRIVE guidelines and EU Directive 2010/63/EU for animal experiments rules were followed in the study.

In order to be anesthetized before surgery, all rats were administered 30 mg/kg ketamine hydrochloride (Ketalar®, Pfizer, Türkiye), and 5 mg/kg Xylazine (Rompun®, Bayer, Türkiye), intramuscularly.

The rats were prepared in a supine position and the abdominal skin was cleaned with a 10% povidone iodine solution after it was shaved. Then, all rats underwent laparotomy with a 2-centimeter incision in the upper midline. Liver suspensory ligament was cut and released. In accordance with the method described by Higgins and Anderson, a 70% hepatectomy was performed by ligating the left lateral and medial lobes of the liver with 4/0 silks from the pedicles at the junctions with the inferior vena cava. After the procedure, all rats were administered 20 mg/kg saline, intraperitoneally. Then, the laparotomy incision was closed with 3/0 silk and the surgical area was cleaned with 10% povidone iodine. The resected liver tissues were weighed and recorded with a precision scale for each rat. After the procedure, rats were allowed to feed and water intake.

Group 1 and Group 3 were administered 0.2 ml of saline, i.v., through the tail vein at 0 and 6th hours after a 70% hepatectomy. Group 2 and Group 4 were administered exogenous VEGF164 (Sigma, St. Louis, USA) at a dose of 500 ng/0.2 ml, i.v., in the same way at 0 and 6th hours. None of the rats died during the study period.

Group 1 and Group 2 were anesthetized after 24 hours, Group 3 and Group 4 after 72 hours, and thereafter laparotomy and sternotomy were applied in all rats. Intracardiac 5 ml blood was collected, and the remnant liver tissue was completely removed, and the rats were sacrificed. The remnant liver tissues resected from each rat were weighed separately on a precision scale and recorded. These liver tissues were then placed in a 10% formalin solution for histopathological examination. Aspartate transaminase (AST), alanine transaminase (ALT), and total bilirubin levels were measured from blood samples collected in all groups.

In order to find the liver regeneration rate, first, the total weight of the initial liver tissue was found by calculating the ratio of liver tissue weight resected by the 70% hepatectomy. Then, as described in the literature, the liver regeneration rate was found with the following formula[8]. In the formula, A is: liver weight obtained during sacrification, B is: initial liver total weight (Initial liver total weight was calculated by proportioning the resected liver weight), C is: resected liver weight.

Liver regeneration rate: $A-(B-C)\times 100$

Blood samples collected from rats were centrifuged at 1500 rpm for 10 minutes, and their plasmas were separated. AST, ALT, and total bilirubin values were analyzed separately for each rat on the Konelab PRIME 60i Clinical Chemistry Analyzer (Thermo SCIENTIFIC, Finland) autoanalyzer.

Tissue samples taken from each rat, after being kept in a 10% formalin solution for 24 hours, underwent histopathological examination. Four-micron-thick sections were taken from the liver tissues placed in paraffin blocks and stained with Hematoxylin Eosin (HE).

Four-micron-thick sections prepared from paraffin blocks were stained on a fully automated immunohistochemistry staining machine (Ventana Medical Systems, Tucson, USA) using the Avidin-biotin peroxidase technique and the "Ventana i-View DAB Detection" kit (Ventana Medical Systems, Tucson, USA) with Ki-67 antibody (Clone SP6, Neomarkers, USA, Benchmark XT). To demonstrate antibody immune reactivity, the Ventana diaminobenzidine (DAB)

system, in which a brown staining appeared, was used. Nuclear staining was accepted as positive staining. In each liver sample, at x400 magnification on a light microscope, 500 hepatocytes were evaluated. The percentage of the number of nuclear positive stained cells was identified in the evaluated hepatocytes.

Statistical analysis of the study was performed using the Statistical Package for the Social Sciences (SPSS) for Windows (15.0 version). The Mann-Whitney U test was used to compare the data obtained from the subjects. Results were evaluated in the 95% confidence interval. $p<0.05$ was considered as significant.

Results

The mean liver regeneration rates in Group 1 and Group 2 were found to be 42.24±13.26% and 64.84±46.30%, respectively. The mean liver regeneration rates of Group 3 and Group 4 were 74.88±12.63% and 82.82±39.41%, respectively. When liver regeneration rates of Group 1 and Group 2 were compared, no statistically significant difference was found. The same situation was observed in Group 3 and Group 4 ($p>0.05$). When liver regeneration rates calculated for Group 1 and Group 3 were compared, a statistically significant difference was observed ($p=0.016$). However, when the liver regeneration rates of Group 2 and Group 4, which were administered VEGF, were compared, no statistically significant difference was found ($p>0.05$, Table 1).

Results of liver function tests are given in Table 2. When the groups were compared in terms of AST values, there was a statistically significant difference between Group 1 and Group 3 and between Group 2 and Group 4 ($p=0.009$). When the groups were compared for total bilirubin levels, a statistically significant difference was found only between Group 2 and Group 4 ($p=0.009$).

In the comparison of the Ki-67 proliferation index between the groups, statistically significant differences were found between Group 1 and Group 3, Group 2 and Group 4, and also between Group 3 and Group 4. Data belonging to the Ki-67 proliferation index are presented in Table 3.

Table 1. Liver Regeneration Rates

	Group 1 n=5 (Mean±SD)	Group 2 n=5 (Mean±SD)	Group 3 n=5 (Mean±SD)	Group 4 n=5 (Mean±SD)	P1	P2	P3	P4
Liver regeneration rate (%)	42.24±13.26	64.84±46.30	74.88±12.63	82.82±39.41	p>0.05	p>0.05	p=0.016	p>0.05

P1: Comparison of Group 1 and Group 2; P2: Comparison of Group 3 and Group 4; P3: Comparison of Group 1 and Group 3; P4: Comparison of Group 2 and Group 4.

Table 2. Liver Function Tests

	Group 1 n=5 (Mean±SD)	Group 2 n=5 (Mean±SD)	Group 3 n=5 (Mean±SD)	Group 4 n=5 (Mean±SD)	P1	P2	P3	P4
AST (U/L)	966.4±451.57	1445.4±252.42	315±124.17	223.6±107.57	p>0.05	p>0.05	p=0.009	p=0.009
ALT (U/L)	368.4±291.6	474.6±199.33	65.2±21.73	111.8±35.22	p>0.05	p=0.009	p=0.009	p=0.009
Total bilirubin (mg/dl)	0.93±0.63	0.96±0.5	0.42±0.15	0.35±0.03	p>0.05	p>0.05	p>0.05	p=0.009

P1: Comparison of Group 1 and Group 2; P2: Comparison of Group 3 and Group 4; P3: Comparison of Group 1 and Group 3; P4: Comparison of Group 2 and Group 4.

Table 3. Ki-67 Proliferation Index Values

	Group 1 n=5 (Mean±SD)	Group 2 n=5 (Mean±SD)	Group 3 n=5 (Mean±SD)	Group 4 n=5 (Mean±SD)	P1	P2	P3	P4
Ki-67 proliferation index	4.4±3.36	2.4±1.67	28.6±10.95	54.6±17.2	p>0.05	p=0.028	p=0.009	p=0.009

P1: Comparison of Group 1 and Group 2; P2: Comparison of Group 3 and Group 4; P3: Comparison of Group 1 and Group 3; P4: Comparison of Group 2 and Group 4.

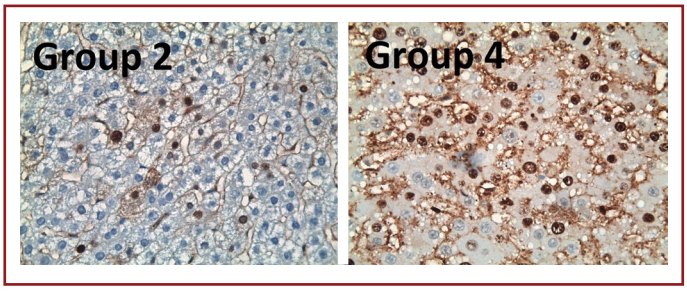

Figure 1. Ki-67 positive area in Group 2 and Group 4 liver tissue (Streptavidin-Biotin, x400).

Discussion

After liver resection, compensatory hyperplasia of hepatocytes occurs rapidly and continues until the liver reaches its original size. Compensatory hyperplasia of the liver is the fastest known tissue growth in mammals[8,9-12]. Ehrenfried et al.[13] stated that resection of 68-70% of the liver is the most suitable resection rate for partial hepatectomy studies in rats. In the literature, it has been stated that the removal of 2/3 of the liver mass in rats is an optimum ratio in terms of regenerative stimulation, and that a partial hepatectomy at the lower or higher values of this ratio may slow the regeneration[9]. In experimental liver resections of rats, only the left lateral lobe is resected for a 30% partial hepatectomy, whereas for a 70% hepatectomy, resection of the median and left lateral lobes by the Higgins and Anderson technique is sufficient[5,9]. In this study, partial hepatectomy was performed at the rate of approximately 70% by resecting the median and left lateral lobes of the liver with the same technique. It is stated that in rats the liver tissue is regenerated almost in 5-7 days after resection[10,14,15]. It has been reported that while there is a very rapid increase in the first 7 days after partial liver transplantation in humans, full regeneration is realized nearly in 3 months[15,16].

In general, the initiation, progression, and control of regeneration in the liver are ensured by all liver cells. The relationship between dividing hepatocytes, non-parenchymal cells (Kupffer cells, endothelial cells, Ito cells), and extracellular substances also plays an important role in regulating regeneration[17]. Studies on the phases of liver regeneration have shown that cytokines and growth factors, although they are not directly related to cell proliferation, activate many genes[18-22]. VEGF is localized on the short arm of chromosome 6 (6p12.3), and its molecular weight is 45 kDa[23]. VEGF, which is one of the growth factors effective in liver regeneration, is the most basic and most important factor in angiogenesis[24,25]. Greene et al.,[26] in a study they performed, have shown that liver regeneration is an angiogenesis-dependent event.

It has been reported that VEGF expression is highly increased during liver regeneration induced by drug intoxication[27]. Namisaki et al.[28] stated that they significantly reduced the mortality rate with VEGF treatment in rats that they chemically induced with acute hepatic insufficiency. Akiyoshi et al.[29] also suggested that serum VEGF levels may be related to the degree of hepatocyte regeneration. It has been shown that VEGF proliferates hepatic sinusoidal cells via vascular endothelial cell growth factor receptor-1 and vascular endothelial cell growth factor receptor-2 over receptors after hepatectomy[30,31]. In the literature, it has been shown that VEGF production in rats after a 70% hepatectomy is evident at 24 hours, peaks at 48-72 hours, and provides endothelial proliferation[6,32].

It has also been shown in regeneration models performed with a 70% hepatectomy that the time period between hepatocyte proliferation and initiation of endothelial cell proliferation is approximately 24-48 hours[32,33]. The utility of the Ki-67 proliferation index as an indicator of cellular proliferation activity has been expressed in several publications[34,35]. In this study, the Ki-67 evaluation of the proliferation index was also conducted as a regeneration criterion. When the Ki-67 proliferation index was compared between Group 1 and Group 2, no statistically significant result was found ($p>0.005$). At the end of 72 hours, when Group 3 and Group 4 were compared in terms of the Ki-67 proliferation index, the proliferation index rate was significantly higher in Group 4 and it was found to be statistically significant ($p=0.028$). This situation showed that exogenous VEGF administration increased liver regeneration after hepatectomy, especially after 24 hours. In the literature, there are studies in support of our results showing that VEGF increases liver cell proliferation[7,8,36]. In this study, Ki-67 positivity is evidently seen in Group 2 and Group 4 in which exogenous VEGF was given, in immunohistochemical staining for evaluation of the Ki-67 proliferation index (Fig. 1).

Liver regeneration rates calculated according to the methods in the literature were statistically compared[37,38]. When the regeneration rates were compared between Group 1 and Group 3, which were not administered VEGF, a statistically significant difference was found ($p=0.016$). However, no statistically significant difference was found in the comparisons of the other groups. In the literature, in a study performed by administering a 30% hepatectomy,

then 200 ng of exogenous VEGF to each rat, the increase in liver weight was examined but no statistically significant result was found[8]. In another study, liver regeneration was evaluated by giving 1000 ng of exogenous VEGF to each rat after a 70% hepatectomy and it was stated that the weight gain in the liver was statistically significant[38]. In our study, 500 ng of exogenous VEGF was given to each rat, but the increase in liver weight was not statistically significant in the groups other than Group 1 and Group 3, where VEGF was not administered. This result suggests that VEGF may have a dose-dependent effect on the mass increase of the liver.

Serum transaminases are highly sensitive to detect hepatocyte damage; regardless of the etiologic factor, serum levels increase in all cases where liver damage persists. In this study, serum AST, ALT, and total bilirubin values were measured to evaluate liver function. When AST and ALT values of Group 1 and Group 3 were compared, these values were found to be significantly lower in Group 3 (p=0.009). Changes in ALT values in Group 3 and Group 4 were found to be statistically significant (p=0.009). A significant improvement was observed in AST, ALT, and total bilirubin values in Group 2 and Group 4 (p=0.009). It has been reported in the literature that exogenous VEGF has a minimal effect on liver biochemical markers[7]. Changes in AST, ALT, and total bilirubin values in this study support that literature.

In conclusion, although the liver regeneration index was higher in VEGF-administered groups, no statistically significant difference was found in this study. However, a significant difference emerged between the groups where no VEGF was administered. This may be due to the increase in liver volumes resulting from VEGF-induced liver regeneration in both groups; it was also considered that it may be due to the dose of VEGF administered as exogenous. It was also thought that the limited number of subjects could be effective in not reaching a statistically significant result. We think the fact that the Ki-67 proliferation index was very high in VEGF-administered sacrificed rats at the 72nd hour supports this consideration. Therefore, it is thought that examining the positive effect of exogenous VEGF administration on liver regeneration through randomized prospective studies may pave the way for clinical applications.

Ethics Committee Approval: Ethics Committee approval was received for the study (2009-50-248). ARRIVE guidelines and EU Directive 2010/63/EU for animal experiments rules were followed in the study.

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