Int J Clin Exp Pathol 2017;10(3):2939-2949
www.ijcep.com /ISSN:1936-2625/IJCEP0043312

Original Article

Local and systemic angiogenic and antiangiogenic response in rats after 70% hepatectomy

Dauren Sarsenov[1], Ahmet Bülent Dogrul[1], Kemal Kosemehmetoglu[2], Mehmet Bulent Tirnaksiz[1], Osman Abbasoglu[1]

Departments of [1]General Surgery, [2]Pathology, Hacettepe University School of Medicine, Hacettepe University Sihhiye Campus, Ankara, Turkey

Received October 27, 2016; Accepted February 7, 2017; Epub March 1, 2017; Published March 15, 2017

Abstract: Angiogenesis accompanying liver regeneration is regulated by both proangiogenic and anti-angiogenic effectors. This study investigated angiogenesis at both local and systemic levels also targeting measurement of endostatin's local expression during hepatic regeneration. Methods: 64 female rats underwent 70% hepatectomy and were sacrificed on the posthepatectomy days 1, 3, 5, 7, 10, 14 and 21. Local angiogenesis and anti-angiogenesis were defined by immunoreactivity to VEGFR-2 and endostatin respectively. Systemic VEGF and endostatin were identified by ELISA with corresponding antibodies. Results: Both VEGF and VEGFR-2 began to rise on day 3. On day 5 VEGF reached local peak with 260.60 pg/ml while VEGFR-2 expression became 3.63 degrees. At day 21 both parameters returned to corresponding basal levels. Peak values of systemic endostatin (68.83 ng/ml) were detected on day 7. Local endostatin expression in tissue was found to be high through groups from 7th to 14th days. On day 14 through 21 when tissue endostatin went decreasing systemic one stayed high. Conclusions: throughout liver regeneration expression of endostatin in extracellular matrix was high and related to angiogenesis. Systemic presence of endostatin was correlating with in situ expression of endostatin which should play a role in regulation of endocrine effects of angiogenic factors like VEGF.

Keywords: Vascular endothelial growth factor, endostatin, angiogenesis, anti-angiogenesis, liver regeneration

Introduction

Angiogenesis plays a pivotal role in many physiologic and pathologic states. Any trauma or, more precisely, alteration leading to tissue loss exploits angiogenesis in physiological limits to provide regeneration. Angiogenesis related factors for many years attract a great attention to local paracrine effects of the process with its possible systemic derivatives [1-9]. In some pathological states angiogenesis is characterized by altered production of effectors which are normally produced on physiological background. Many studies were focused on discovering the principal role of angiogenesis and factors playing fundamental role in processes accompanying tumorigenesis, metastasis formation, some chronic and progressive connective tissue diseases [10-16].

Studies of angiogenesis established a range of methodologies for experimental modelling of angiogenesis. One of most reproducible and easily performed methodologies is angiogenesis model on liver regeneration model in rodent species. The very process of liver regeneration being a multifactorial in nature is shown to be angiogenesis dependent process [17-22]. Regulation of liver regeneration together with regulation of angiogenesis taking place during liver regeneration has both auto- and paracrine pathways along with endocrine ones. This process emphasizes a universal mechanism of neutralizing effects of outer world on the inner world providing homeostasis and showing almost infinite reserve capacity of the liver. Evolutionary background of this function is substantiated by possibility of damage to liver by aggressive and potentially harmful agents entering enteral system which consequently leads to hyperplasia and proliferation [17, 18, 23-26].

The measure of hepatic proliferation is thought to be dependent on liver-body ratio. It is well known that liver maintains proliferation till met-

abolic demand of the body is provided [17, 18, 21, 23-27]. In its turn, angiogenesis is induced due to hypoxic hepatocytes suffering from growing oxygen demand in parenchymal islets isolated from normal vasculature. Being invasive in nature angiogenic pathway is up-regulated by a variety of factors with major contribution of VEGF ligand produced by hypoxic pool of hepatocytes [28-31]; therefore switching endotheliocytes' extravasation on; which provides degradation of extracellular matrix by means of expression of various types of MMP family proteinases [32-35]. Dogrul et al. in their study showed that during liver regeneration, cessation of regeneration process was related to anti-angiogenic activity which was evaluated using RT-PCR detection of collagen-18 mRNA [36]. Levels of collagen-18 mRNA were peaking on day 10 having progressive lowering down through day 14. Angiogenesis driven proteolytic matrix degradation results in proteolytic cleavage of collagen-18 yielding in production of endostatin shown to have potent anti-angiogenic effect [17, 18, 21, 23, 24, 26, 28, 37-39].

The aim of this study was to evaluate local expression of endostatin in liver during regeneration and systemic effect of local angiogenesis in rats after 70% hepatectomy setting the link between local markers of angiogenesis and anti-angiogenesis and systemic ones.

Materials and methods

Sixty four Sprague-Dawley female rats weighing around 230-320 g were included in study and were fed with standard diet and water ad libitum and kept under standard room temperature of 22-24°C and in 12 hour long circadian cycle. The study was approved by Institutional Committee of Ethics in Animal Research under №2012/6-1 in accordance with Institutional Declaration and "Helsinki Declaration on Use of Laboratory Animals". Study's funding was provided by Scientific Research Unit of Hacettepe University (project number 012 D06 101 013).

Experimental design

Animals were distributed into 8 groups. Groups were numbered after day of sacrification (sham group = 0). Hepatectomy was performed according to procedure described by Higgins and Anderson [40] under general anaesthesia with Ketalar (5% ketamine hydrochloride) and Alfazyne (2% xylazine). Sacrification was performed by inducing general anaesthesia followed by desanguination for obtaining blood specimen (5-10 ml). Extirpated livers which underwent regeneration were placed in 10% formaldehyde and kept for 1-2 days. Tissue blocks were obtained by selecting and cutting widest portions of grossly regenerated liver lobes. Paraffin embedded sections were cut for 4 μm thickness.

Blood samples were frozen after centrifugation at 1000 rpm for 4 minutes, whereupon all upcoming serum samples were kept under -80°C until obtaining all blood samples from all groups sacrificed.

Immunohistochemical evaluations were performed using standard staining protocols. Staining was applied on formalin fixed tissue blocks embedded in paraffin cut in 4 μm sections. Antigen retrieval was done in boiling citrate buffer (pH = 6.0). After incubation with primary antibodies sections were subjected for biotin-streptavidin-DAB (Acu-Stain™ Mouse + Rabbit HRP Kit (Genemed Biotechnologie, 458 Carlton Court, South San Francisco, CA 94080) and Mouse specific HRP/DAB detection IHC Kit (Abcam PLC, 330 Cambridge Science Park, Cambridge, United Kingdom (ab64259) in case of endostatin antibodies) sequence according to manufacturer's instructions.

Evaluation of regeneration

Regeneration of liver was evaluated according to immunoreactivity to PCNA (NeoMakers (Cat. #MS-106-P), Labvision Corporation, Fremont, California, ABD). Nuclei stained in brown were evaluated as positive and were counted under ×40 magnification among 1000 cells giving proportion in percents.

VEGFR-2 expression

Angiogenic activity was assessed by immunoreactivity to VEGFR-2 (Hemangioblast marker - ab2349, 500 μl; rabbit polyclonal; anti-Rat, Abcam PLC, Cambridge, UK). Sections were evaluated according to cells having brown staining and counted as number of rows formed around vessels' cross-sections including portal and central veins. Degree of expression was calculated as an average through whole speci-

men. One row of positive cells around vessels in average was accepted as +1. Average of 2 rows was accepted as +2, and 3 rows as +3.

Anti-angiogenesis assessment

Immunoreactivity to Abcam Anti-Endostatin antibody (Anti-Endostatin antibody [4i37] -ab64-569, 100 µl at 0.1 mg/ml; Mouse monoclonal; anti-Rat, Abcam PLC, Cambridge, UK) was assessed. Sections were assessed under ×40 magnification detecting brown staining in perisinusoidal spaces. Estimation was performed according to the following scale: very low intensity staining in all fields or mid intensity staining in one field - +1; mid intensity in all fields or high intensity staining in a single field - +2; homogenous high intensity staining in all fields - +3; very high intensity staining in all fields - +4. Evaluated fields were well away from fields of necrosis, edges and artefacts.

Defining systemic angiogenesis parameters

Systemic angiogenesis parameters were evaluated by immunoreactivity to anti-VEGF antibodies using VEGF Rat ELISA Kit (ab100787 - 1×96 Well Plate, Abcam PLC, 330 Cambridge Science Park, Cambridge CB4 0FL, United Kingdom). Previously centrifugated blood samples were stored at -80°C. After defreezing all collected samples were processed at once. Standard solution was prepared according to manufacturer's instructions with serial dilution giving 10 plates with concentration range of 0-500 pg/ml. Detection antibody in amount of 100 µl was diluted with distilled water up to 80 times. Hundred µl HRP-streptavidin were diluted with 12 ml of assay diluent (giving 120 times dilution). Detection antibody was incubated with sample serum and standard solution under room temperature for 2.5 hours. Serial washing with biotin and consequent streptavidin incubation lasted for 1 hour and 45 minutes respectively under room temperature. Tetramethylbenzidine (TMB) was used as staining agent and was added to plates and left under room temperature in darkness. Thereafter, stop solution was added in amount of 50 µl and processed in spectrophotometer under 450 nm wavelengths.

Defining systemic anti-angiogenesis parameters

Systemic level of endostatin was defined using Rat ES ELISA kit (CSB-E07975r - 1×96 Well Plate, Cusabio PLC, Incubator Building 4th floor, Wuhan University Science Park, No.te-1, Daxueyuan Road, Donghu Hi-Tech Development Area, Wuhan, Hubei Province 430223, P. R. China). Previously centrifugated blood samples were stored at -80°C. After defreezing all collected samples were processed at once. Basic standard solution was prepared according to manufacturer's instructions after 30 seconds centrifuging under 10000 rpm and adding 1 ml of sample diluent forming stock solution. Further serial dilution gave 8 plates with concentration range of 0-400 ng/ml. Detection antibody in amount of 100 µl was diluted with distilled water up to 80 times. Hundred µl HRP-streptavidin were diluted with 12 ml of assay diluent (giving 120 times dilution). Detection antibody was incubated with sample serum and standard solution under room temperature for 2.5 hours. Serial washing with biotin and consequent streptavidin incubation lasted for 1 hour and 45 minutes respectively under room temperature. Tetramethylbenzidine (TMB) was used as staining agent and was added to plates and left under room temperature in darkness. Thereafter, stop solution was added in amount of 50 µl and processed in spectrophotometer under 450 nm.

Statistical analysis

SPSS package 20 (IBM Corp.) was used for statistical analysis.

Variables were investigated using visual (histograms, probability plots) and analytical methods (Kolmogorov-Smirnov/Shapiro-Wilk's test) to determine whether or not they were normally distributed.

Difference between parametric variables was analysed using one way ANOVA test. Differences between ordinal variables were assessed using Kruskal-Wallis and Mann-Whitney U test.

Correlation analysis of systemic and local markers of angiogenesis and anti-angiogenesis was performed assessing graphic data and using Pearson's test.

In-situ expression of VEGFR-2 and endostatin was assessed using standard technique for evaluation of potentially non-quantifiable visual data gathered from immunohistochemical microimages. Due to the fact that visual assessment of immunohistochemical images

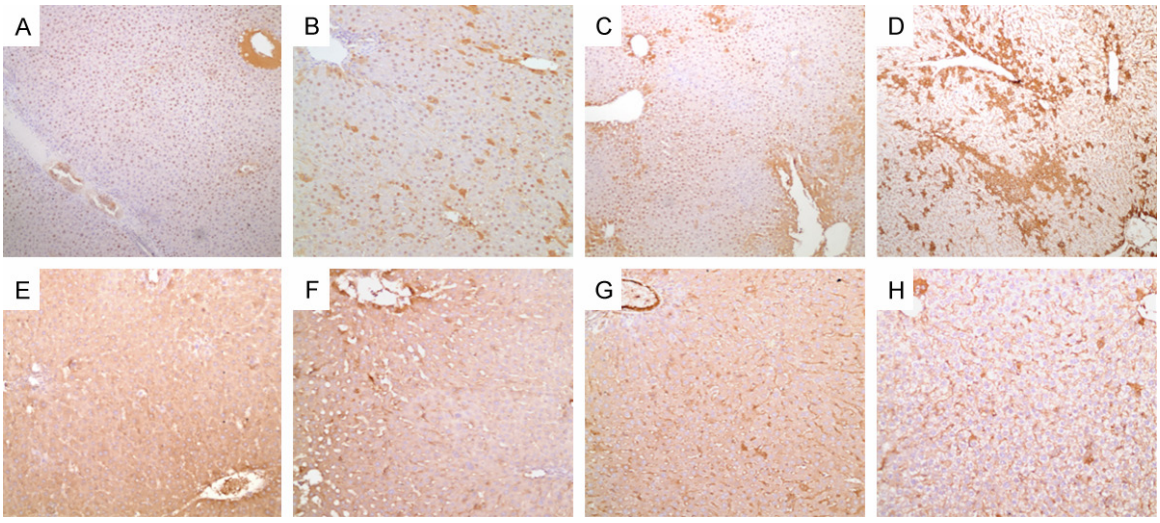

Figure 1. Upper row - local VEGFR-2 expression; A. Absence of expression; B. Low expression; C. Moderate expression; D. High expression. Lower row - local expression of endostatin; E. Low expression; F. Moderate expression; G. High expression; H. Very high expression.

can give pseudocontinuous integer-based spectrum of values (0-4) VEGF and endostatin expression was assumed to be parametric (not ordinal) and delt respectively (**Figure 1**). Significance level was accepted as $P \leq 0.05$.

Results

Regeneration

According to sham group first day's group showed statistically significant level of expression with mean of 57.2% ($P < 0.05$). Third day group also showed statistically significant level of expression with mean of 28.0% ($P < 0.05$), while fifth day expression with 5.15% was assumed to return to basal proliferation activity ($P = 0.87$).

Local angiogenesis

According to sham group having mean expression of VEGFR-2 of 5.13 units 1st day group had significant decrease in expression with 2.3 units ($P = 0.005$), 3rd day expression with 2.6 units was also significantly different ($P = 0.02$). On fifth day VEGFR-2 having expression level of 3.6 and having $P = 0.13$ after which 7th day showed lowest expression of 1.6 with $P = 0.02$. In consequent 10th day's group expression started to grow (3.3 units) showing insignificant difference having $P = 0.08$, while expression on 14th day was 2.6 with $P = 0.07$. Finally VEGFR-2 expression went back to basal levels with 4.1 units ($P = 0.34$).

Local anti-angiogenesis

Basal level of endostatin expression in tissue in sham group was high with mean of 3. First day group showed mean expression of 2 with $P = 0.08$, on third and fifth day expression was 2 and 1 respectively with *P* values of 0.04 and 0.007 respectively. On 7th day expression showed level of 2 with $P = 0.07$. On day 10 and 14 expression was high (2 units) with *P* values of 0.40 and 0.44 respectively and finally expression on day 21 had mean of 1 with $P = 0.08$.

Systemic angiogenesis

Systemic levels of VEGF ligand were evaluated in serum. Sham group's levels were accepted as basal (mean concentration = 212.39 pg/ml). On 1st day level was 277.61 with $P = 0.21$, third day mean level was 202.84 with $P = 1.00$, fifth day mean level of VEGF was 260.60 with $P = 0.51$. Seventh day mean level was 242.73 pg/ml with $P = 0.90$, while 10th and 14th days' groups were having mean levels of 230.23 and 275.05 pg/ml respectively with *P* values of 0.99 and 0.25 respectively. Twenty first day mean level was 230.36 pg/ml with $P = 0.99$.

Systemic anti-angiogenesis

Systemic endostatin levels taken as mean concentration through groups were assessed according to the sham group (11.18 ng/ml) evaluating statistical significance as well. First day

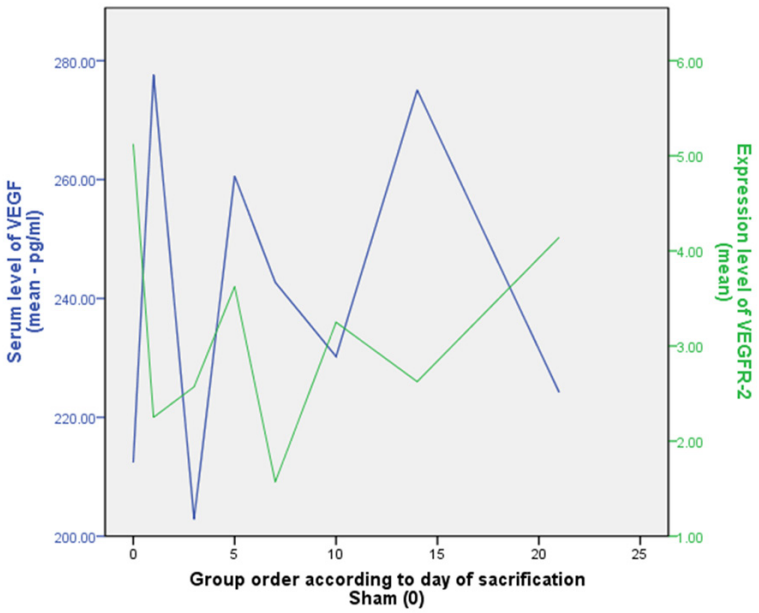

Figure 2. Note day 5, 7, 10 and 14th trends where we can observe initial overexpression of VEGF with accompanying rise in VEGFR-2 expression which leads to consequent saturation of VEGF-receptors with VEGF reflecting day 7th local minimum. Day 10th decrease in circulating VEGF demonstrates angiogenesis' downregulation due to anticipated decrease of hypoxic cell pool. The latter, in turn makes VEGFR-2 gradually release VEGF into systemic circulation with consequent metabolization until pre-angiogenesis systemic equilibrium is established.

group had mean of 36.15 ng/ml (P = 0.50), third day group had mean concentration of 18.90 ng/ml (P = 0.99). Fifth day mean was 53.24 ng/ml (P = 0.07), while actual peak value was observed on day 7 when mean concentration was 68.83 ng/ml (P = 0.008). On day 10 concentration was still high with 63.24 ng/ml with P = 0.014 and on day 14 mean concentration decreased to the level of 37.85 ng/ml (P = 0.43). On 21st day mean became 38.72 ng/ml with insignificant difference (P = 0.40).

Correlation between local and systemic angiogenesis

A calculation of correlation between local and systemic angiogenesis was performed. Pearson's test for correlation failed to reveal statistically strong correlation. On day 1 VEGF concentration rising to 277.61 ng/ml being opposed to 2.25 units of expression for VEGFR-2 shows weak correlation without statistical significance (ρ = 0.074; P = 0.86). Further oscillations of VEGF concentration and VEGFR-2 expression showed weak negative and statistically insignificant correlation (ρ = -0.22; P = 0.64) on day 3 and day 5 (ρ = -0.37; P = 0.37). Day 7 group came up with positive correlation (ρ = 0.31; P = 0.50) whereupon on days 7 and 10 negative correlation again showed up (ρ = -0.18; P = 0.67 and ρ = -0.49; P = 0.22 respectively). Last point of intersection showed positive intermediate correlation power still being statistically insignificant (ρ = 0.52; P = 0.23). Graphic interpretation relating to spoken points showed anticipated distribution curves for VEGF ligand concentration binding to investigated receptor VEGFR-2 making it unavailable to working antibodies (**Figure 2**).

Correlation between local and systemic anti-angiogenesis

Correlation between local and systemic anti-angiogenesis was done between endostatin in-site and serum endostatin and endostatin-like ligands. This kinetics was expected to be parallel due to the fact that endostatin after expression of collagen-18 and its sequential proteolytic cleavage should be washed out to systemic blood flow giving delayed correlation with curves of systemic endostatin following local one with slide of the curve to the right (**Figure 3**).

On day 1 correlation showed to be negative and weak (ρ = -0.055; P = 0.90) after that on day 3 correlation got stronger (ρ = -0.67; P = 0.10). Through days 5, 7 and 10 correlation was mostly weak without being statistically significant (ρ =0.14; P = 0.74, ρ = 0.06; P = 0.91, ρ = 0.44; P = 0.33 respectively). On days 14 and 21 correlation became negative without statistical significance (ρ = -0.05; P = 0.91 and ρ = -0.25; P = 0.54 respectively). Graphic interpretation of correlation is much more confining showing systemic kinetics being similar to local expression graph (**Figure 3**). Analysis of graphic correlation shows the general trend of interrelationship seeming pretty direct.

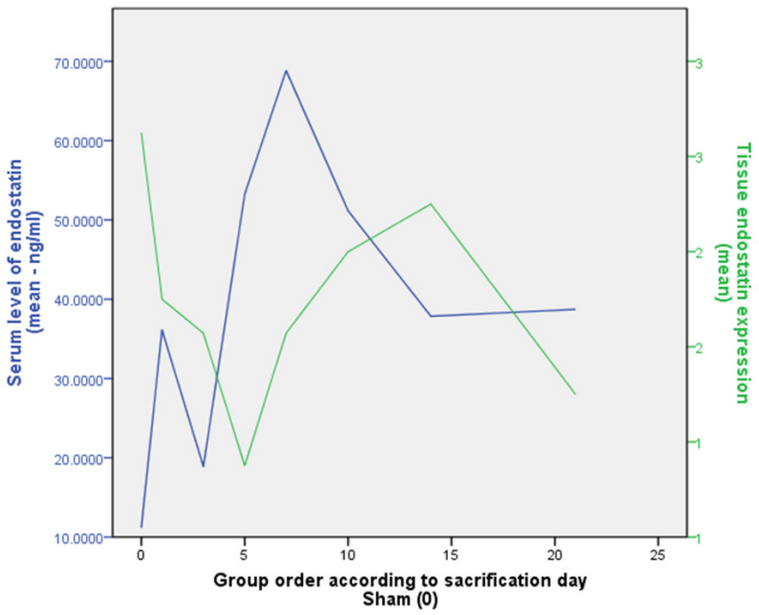

Figure 3. Please note systemic endostatin rapidly increasing in systemic circulation which is supposed to be related to surgical trauma on the day 1 leading to depletion of tissue endostatin as systemic grows through days 1, 3 and 5. Between days 5 and 7 in-situ endostatin production becomes upregulated by angiogenesis contributing to even more exaggerated rise in systemic endostatin concentration persisting throughout days 7-10. Days 14 and 21 show signs of prolonged systemic activity of endostatin which could be explained by necessity for endocrine downregulator of pro-angiogenic activity driven by systemic VEGF.

VEGF and endostatin correlation

Correlation between VEGF and endostatin in systemic blood was also performed exploiting Pearson's test (**Table 1**). First day reveals strong negative correlation close to being significant (ρ = -0.69; P = 0.06) becoming insignificant on days 3 and 5 (ρ = -0.36; P = 0.43, ρ = 0.43; P = 0.29 respectively). Day 7 reveals strong negative correlation being also statistically significant (ρ = -0.85; P = 0.02). Day 10 comes with intermediate correlation power becoming insignificant (ρ = -0.62; P = 0.10). On days 14 and 21 correlation again becoming significant and strong negative (ρ = -0.83; P = 0.01, ρ = -0.98; P < 0.001). Analysis of graphic correlation (**Figure 4**) is performed in the discussion part.

Discussion

Partial hepatectomy in rats is a well known and widely investigated model for angiogenesis studies. Regeneration process starting immediately after hepatectomy mostly depends on expression of promoters of hepatic proliferative pool. Our study's timeline was normalised over regeneration landmarks which finally showed anticipated regeneration similar to that of other studies on this subject with rates which were peaking in between 24-48th hours and getting to basal level on day 7 [24, 36, 41, 42].

It was shown that during angiogenesis hepatocytes form islets composed of newly proliferating mass of cells which start to produce VEGF to upregulate endotheliocytes' invasion into extracellular matrix [21, 26, 29, 30, 43]. In our study angiogenesis tracked by change in VEGFR-2 receptor's local expression and systemic change in VEGF ligand concentration, showed to obey a non-linear dynamics. Basal VEGFR-2 tissue expression was detected to be high, possibly defining high basal expression on endotheliocytes' membranes seen in sham group. VEGFR-2 expression was then dramatically decreasing on day 1 probably reflecting binding of some VEGF ligands which show a local peak of concentration at the same time, possibly due to surgical trauma as it was shown in studies by Saitou Y et al. And Dogrul AB et al. [20, 36], thus changing conformation of receptors hiding VEGFR-2 sites from immunostaining antibodies (**Figure 2**).

As angiogenesis starts to gain momentum on day 3 we can see VEGFR-2 expression rising, while systemic marker of angiogenesis, VEGF hits local minimum. After that on day 5 VEGF and VEGFR-2 have simultaneous peak of their expression which can be explained in two ways: first is that hypoxia drive slows down and VEGFR-2 expression ceases and receptors get freed from VEGF ligands which in turn contributes in simultaneous systemic VEGF level rise; and the second way to explain this phenomenon is that hypoxia drive gets to its maximum resulting in highest expression of VEGFR-2 receptors (throughout the experimental timeline, excluding basal level) and highest level of

Table 1. Correlation of systemic VEGF and endostatin

	Sham	1st day	3rd day	5th day	7th day	10th day	14th day	21st day
Serum VEGF (mean)	212.39	277.61	202.84	260.60	242.73	230.23	275.05	230.36
Serum endostatin (mean)	11.18	36.15	18.90	53.24	68.83	63.24	37.85	38.72
Correlation coefficient (ρ)*	0.24	-0.69**	-0.36	0.43	-0.85**	-0.62	-0.83**	-0.98**
P-value	0.57	0.06	0.43	0.29	0.02	0.10	0.01	< 0.001

*Pearson's correlation rho (ρ) calculated for each group. **Statistically significant strong negative correlation.

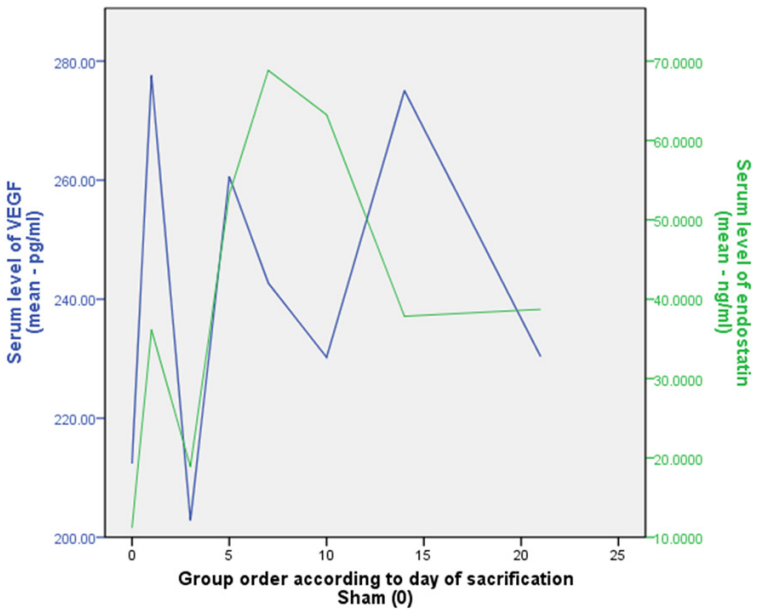

Figure 4. Concluding all above figures this figure demonstrates how expression of pro-angiogenic factor (VEGF) leads to consequent upregulation of matrix-derived antiangiogenic factor (endostatin) forming trends perfectly opposite to each other since the day 5 through 7, 10 and 14. Correlation analysis using Pearson's rho showed statistically significant strong negative correlation between serum VEGF and endostatin on days 1, 7, 14 and 21.

VEGF systemic concentration providing high rate of angiogenesis. The latter seems to be verified by simultaneous rise of endostatin curves for local and systemic expression probably meaning that angiogenesis process came to its maximum invasiveness leading to corresponding expression of endostatin (**Figure 3**). Indeed, studies by Michalopoulos et al., Taniguchi et al., Dogrul et al. show that angiogenesis really does peak throughout 1-3rd days of regeneration [36, 44, 45]. Day 7 brought lowest value for expression of VEGFR-2 together with descending concentration of systemic VEGF showing either down-regulation of angiogenesis or total occupation of aggregate pool of binding sites of VEGFR-2 receptors (**Figure 2**). However, down-regulation seems to be more likely to be recruited due to possible effect of high level of endostatin expression, known to strongly inhibit endothelial cell migration and expression of VEGFR-2 [12, 15, 35, 46-50] (**Figure 4**). Thereafter, on days 10 through 14, level of expression of VEGFR-2 starts rising and on day 10 corresponds to systemic VEGF level reaching local minimum where some turn point takes place. After day 10 local expression of VEGFR-2 is rising up to the day 21 reaching its actual basal level, when VEGF in its turn states its third maximum on day 14 for some not clear reason. We think it could be explained by those VEGF ligands detaching from binding sites of VEGFR-2 receptor pool. On day 21 both receptor and VEGF are trending to reach initial levels of expression.

Anti-angiogenesis in situ showed similarity to earlier studies showing same dynamics as did collagen-18 expression earlier processed using PCR-RT protocols. Earlier spoken study by Dogrul et al. [36] did actually show the level of mRNA expression without showing the real ratio of mRNA translated into collagen-18 which finally was reaching its target destination in ECM. Our study was not considered to reveal the rate of mRNA conversion into endostatin, but to show relative amount of expressed endostatin. There are many ways to actually track the rate of m-RNA translation into collagen-18 finally yielding endostatin. Some of them are quantifying, others are relative, only showing the correlation between production of end-product (here, endostatin) and collagen-18 m-RNA. The reason why we did not prefer quanti-

fying method for detection of tissue endostatin in our study, was the natural limitation of the only available in our institution method for protein quantification - Western blot, which works with disrupted tissue lysate. And since we decided to specifically detect tissue expression of endostatin in liver we preferred immunohistochemical staining of sample section with further correlation of detected expression rates with the time scale of the experiment (groups according to the day of sacrification).

Dynamics of endostatin tissue staining was compatible with findings of initial washing out to systemic blood flow proved by systemic increase of endostatin on day 1 through 3. On day 3 through 5 local and systemic endostatin showed antiparallel dynamics when local endostatin expression was decreasing, while systemic continued to rise. On day 5 local expression of endostatin due to assumed (ever-rising activation of angiogenesis) intensive local proteolysis must be high, but we can see that endostatin levels are not high at all. Instead we have low levels of local endostatin and high levels of systemic one which could be simply explained by intensive washing out of endostatin from impaired extracellular matrix into systemic bloodstream. Rates of collagen-18 mRNA expression in study by Dogrul et al., did actually show similar dynamics for days 5 through 7 being low until day 10 where collagen-18 mRNA expression rised to form most prominent peak [36]. They showed the actual level of production of mRNA's but not the real expression of collagen-18 as it would be clearer to see the amount of collagen-18 produced. So, there must be some equilibrium between *de novo* production of collagen-18 and washing out of endostatin yielding from proteolytic cleavage of collagen-18, which is perfectly confirmed by high level of systemic endostatin. We think that this parallel rise in both systemic VEGF and systemic endostatin is fitting the concept of negative feedback system aiming to maintain local processes without impairing systemic homeostasis. Thus, when we have angiogenesis in its most invasive and aggressive phase, we just have to have high level of by-product of the very process of angiogenesis, which is endostatin avoiding systemic upregulation of neoangiogenesis to prevent remote side effects of VEGF [7, 11].

To better substantiate latter considerations, here, we have to remind well known principle in enzyme kinetics - generally the product of reversible reaction becomes inhibitor of primary reaction [51]. Endostatin here appears to be the by-product of proteolytic cleavage during matrix degradation [52]. So, here is another consideration - to find a negative feedback regulator of some process one has to look at the "meaning" of the process. That means, in our case, that extracellular matrix being degraded by MMP's, it is matrix which is virtually resisting invasive character of angiogenesis, thus constantly producing by-products capable of inhibiting angiogenesis-related or angiogenesis-driven processes. And, as a consequence, those products must be matrix main composers related products, just as we see endostatin deriving from collagen, angiostatin from plasmin, etc. [53, 54]. Systemic endostatin on day 7 comes to maximum level probably opposing to newly breaking peak of angiogenesis thereafter (VEGF) bending downwards (days 5 through 7 - **Figure 4**). Consequently local endostatin starts gaining mass due to combined effect of washout and progressively decreasing proteolytic cleavage. On day 10 through 21 systemic endostatin trends down but not actually reaches sham's level which corresponds to earlier studies by Colakoglu et al. and Dogrul et al. [36, 41]. Local endostatin on day 10 through 21 shows process of restoring in-tissue depot peaking on day 14 and decreasing by some factor on day 21. Statistical analysis of Pearson's correlation between local and systemic endostatin does give statistically significant ratios.

In contrast to above spoken correlations; the correlation between systemic VEGF and endostatin in our study showed great concordance both statistically and graphically. Correlation analysis using Pearson's rho showed statistically significant strong negative correlation between serum VEGF and endostatin on days 1, 7, 14 and 21. In other words increase in VEGF serum concentration will mean decrease in endostatin serum concentration and vice versa. Being interconnected both angiogenesis and anti-angiogenesis were represented by VEGF and endostatin in serum with negative correlation on certain days of the experiment. Analysing the timeline of the experiment we can see that first day peak was observed for both VEGF and endostatin relating to surgical trauma as other studies assume [19, 20, 36]. Day three came out with significant decrease for both parameters which can be explained by starting liver regeneration but still without sig-

nificant pool of cells suffering from hypoxia; thus not serving as a reason for up-regulation of VEGF expression. Day 5's systemic peak of VEGF corresponds to rising level of endostatin showing that VEGF being a derivative of local hypoxia and driving proteolytic cleavage of collagen-18 is providing high systemic level of endostatin which is still going high even after VEGF starting to go down on days 7 through 10. On day 14 we can see a local maximum for VEGF which is actually to be controlled on the matter of being an artefact (further investigations needed). So, summing up we just came to a new way of evaluating angiogenesis. All conditions related to angiogenesis up-regulation were assessed in previous studies using only one link of angiogenesis chain, namely either VEGF or endostatin thus leading to a lack of productivity/reproducibility when they were trying to evaluate the predictive meaning of spoken systemic angiogenesis effectors. Our calculations show that when we assess angiogenesis with two consequent and universally expressed steps of the same process more precise results can be achieved, which, of course is to be studied and verified in further research.

Some researchers like Schips et al. in their clinical study on renal cell cancer (RCC) patients were trying to determine if VEGF and endostatin would be a good predictor of survival in RCC patients. There was no statistically significant difference between RCC patients and control groups in terms of VEGF and no difference in terms of endostatin levels between spoken groups at all. No correlation (Spearman's rho) was revealed between levels of VEGF and endostatin among RCC patients [2]. Both parameters failed to show any prognostic significance in predicting survival. In other study by Teh et al. we can see population composed of breast carcinoma patients who were found to have statistically significant decrease in tumor vascularisation in group having higher preoperative systemic endostatin levels [7]. This kind of studies shows correlation between invasiveness of angiogenesis (read; local changes) and systemic levels of endostatin as a representative of angiogenesis. Zhang et al., in their study tried to reveal if VEGF and endostatin can be of any meaning for differentiation of benign and malignant pathologic processes. They showed that patients with pleural effusions further (post factum) revealed to be of malignant origin had significantly higher serum levels of VEGF and endostatin. Authors also state that combination of VEGF/endostatin and CEA measurements together can significantly increase the value of diagnostic pleural effusion analysis [3]. It is clear that clinical studies need reliable markers of angiogenesis. Our study demonstrates the possibility for elaboration of such a marker based on evaluation of systemic angiogenic and anti-angiogenic factors.

Conclusion

Local expression of endostatin during angiogenesis model demonstrated its high accordance with earlier estimations of collagen-18 mRNA expression throughout timeline of liver regeneration in rats. Systemic endostatin should be playing important role in endocrine inhibition of systemic angiogenesis staying highly expressed in blood serum at the late stages of the liver regeneration. And finally, antagonistically working factors like VEGF and endostatin both originating from the same process can be mutually calibrated in order to obtain a more verifiable marker for systemic evaluation of angiogenesis.

Acknowledgements

Authors would like to thank Mr. Mutlu Hayran for his precious contributions in statistical analysis of the study. We thank the great teams of Experimental Animal and Clinical Biochemical Laboratories of the University of Hacettepe for their never ending support. We also thank the crew of the Pathology Laboratory for their attitude and dedication which as a result saved us a great amount of time while working on specimen preparation and microscopy.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Dauren Sarsenov, Department of General Surgery, Hacettepe University School of Medicine, Hacettepe University Sihhiye Campus, Ankara 06000, Turkey. Tel: +90 555 680 08 56; Fax: +90 212 375 6812; E-mail: dr.dauren.sarsenov@gmail.com

References

[1] Poon RT, Ho JW, Tong CS, Lau C, Ng IO, Fan ST. Prognostic significance of serum vascular en-

dothelial growth factor and endostatin in patients with hepatocellular carcinoma. Br J Surg 2004; 91: 1354-1360.
[2] Schips L, Dalpiaz O, Lipsky K, Langner C, Rehak P, Puerstner P, Pummer K and Zigeuner R. Serum levels of vascular endothelial growth factor (VEGF) and endostatin in renal cell carcinoma patients compared to a control group. Eur Urol 2007; 51: 168-174.
[3] Zhang Y, Yu LK and Xia N. Evaluation of serum and pleural levels of endostatin and vascular epithelial growth factor in lung cancer patients with pleural effusion. Asian Pac J Trop Med 2012; 5: 239-242.
[4] Dhar DK, Ono T, Yamanoi A, Soda Y, Yamaguchi E, Rahman MA, Kohno H and Nagasue N. Serum endostatin predicts tumor vascularity in hepatocellular carcinoma. Cancer 2002; 95: 2188-2195.
[5] Mitsuma W, Kodama M, Hanawa H, Ito M, Ramadan MM, Hirono S, Obata H, Okada S, Sanada F, Yanagawa T, Kashimura T, Fuse K, Tanabe N and Aizawa Y. Serum endostatin in the coronary circulation of patients with coronary heart disease and its relation to coronary collateral formation. Am J Cardiol 2007; 99: 494-498.
[6] Standker L, Schrader M, Kanse SM, Jurgens M, Forssmann WG and Preissner KT. Isolation and characterization of the circulating form of human endostatin. FEBS Lett 1997; 420: 129-133.
[7] Teh SH, Hill AD, Lee AW, Foley D, Kennedy S, Young L, McDermott E and O'Higgins N. Raised plasma endostatin levels correlate inversely with breast cancer angiogenesis. J Surg Res 2004; 116: 165-171.
[8] Peeters CF, de Geus LF, Westphal JR, de Waal RM, Ruiter DJ, Wobbes T, Oyen WJ and Ruers TJ. Decrease in circulating anti-angiogenic factors (angiostatin and endostatin) after surgical removal of primary colorectal carcinoma coincides with increased metabolic activity of liver metastases. Surgery 2005; 137: 246-249.
[9] Clement B, Musso O, Lietard J and Theret N. Homeostatic control of angiogenesis: a newly identified function of the liver? Hepatology 1999; 29: 621-623.
[10] Folkman J. Angiogenesis-dependent diseases. Semin Oncol 2001; 28: 536-542.
[11] Folkman J. Role of angiogenesis in tumor growth and metastasis. Semin Oncol 2002; 29: 15-18.
[12] Jain RK. Tumor angiogenesis and accessibility: role of vascular endothelial growth factor. Semin Oncol 2002; 29: 3-9.
[13] Zent R and Pozzi A. Angiogenesis in diabetic nephropathy. Semin Nephrol 2007; 27: 161-171.
[14] Tolstanova G, Deng X, Khomenko T, Garg P, Paunovic B, Chen L, Sitaraman SV, Shiloach J, Szabo S and Sandor Z. Role of anti-angiogenic factor endostatin in the pathogenesis of experimental ulcerative colitis. Life Sci 2011; 88: 74-81.
[15] Benezra R and Rafii S. Endostatin's endpoints-Deciphering the endostatin antiangiogenic pathway. Cancer Cell 2004; 5: 205-206.
[16] Ylä-Herttuala S, Rissanen TT, Vajanto I and Hartikainen J. Vascular endothelial growth factors: biology and current status of clinical applications in cardiovascular medicine. J Am Coll Cardiol 2007; 49: 1015-1026.
[17] Drixler TA, Vogten MJ, Ritchie ED, van Vroonhoven TJ, Gebbink MF, Voest EE, Borel Rinkes IH. Liver regeneration is an angiogenesis-associated phenomenon. Ann Surg 2002; 236: 703-711.
[18] Michalopoulos GK. Liver regeneration: alternative epithelial pathways. Int J Biochem Cell Biol 2011; 43: 173-179.
[19] Reynaert H, Chavez M, Geerts A. Vascular endothelial growth factor and liver regeneration. J Hepatol 2001; 34: 759-761.
[20] Saitou Y, Shiraki K, Yamaguchi Y, Nakano T, Mizuno S and Uemoto S. Serum vascular endothelial growth factor-receptor 1 during liver regeneration. J Hepatol 2004; 41: 170-171.
[21] Uda Y, Hirano T, Son G, Iimuro Y, Uyama N, Yamanaka J, Mori A, Arii S and Fujimoto J. Angiogenesis is crucial for liver regeneration after partial hepatectomy. Surgery 2013; 153: 70-7.
[22] Fernández M, Semela D, Bruix J, Colle I, Pinzani M and Bosch J. Angiogenesis in liver disease. J Hepatol 2009; 50: 604-620.
[23] Black D, Lyman S, Heider TR and Behrns KE. Molecular and cellular features of hepatic regeneration. J Surg Res 2004; 117: 306-315.
[24] Fausto N. Liver regeneration. J Hepatol 2000; 32 Suppl 1: 19-31.
[25] Furchtgott LA, Chow CC and Periwal V. A model of liver regeneration. Biophys J 2009; 96: 3926-3935.
[26] Pahlavan PS, Feldmann RE Jr, Zavos C and Kountouras J. Prometheus' challenge: molecular, cellular and systemic aspects of liver regeneration. J Surg Res 2006; 134: 238-251.
[27] Kam I, Lynch S, Svanas G, Todo S, Polimeno L, Francavilla A, Penkrot RJ, Takaya S, Ericzon BG, Starzl TE, et al. Evidence that host size determines liver size: studies in dogs receiving orthotopic liver transplants. Hepatology 1987; 7: 362-366.
[28] Cross MJ, Dixelius J, Matsumoto T and Claesson-Welsh L. VEGF-receptor signal transduction. Trends Biochem Sci 2003; 28: 488-494.
[29] Parikh AA and Ellis LM. The vascular endothelial growth factor family and its receptors. Hematol Oncol Clin North Am 2004; 18: 951-971.
[30] Rahimi N. Vascular endothelial growth factor receptors: molecular mechanisms of activa-

tion and therapeutic potentials. Exp Eye Res 2006; 83: 1005-1016.
[31] Toi M, Matsumoto T and Bando H. Vascular endothelial growth factor: its prognostic, predictive, and therapeutic implications. Lancet Oncol 2001; 2: 667-673.
[32] Foda HD and Zucker S. Matrix metalloproteinases in cancer invasion, metastasis and angiogenesis. Drug Discov Today 2001; 6: 478-482.
[33] Gorman JL, Ispanovic E and Haas TL. Regulation of matrix metalloproteinase expression. Drug Discovery Today: Disease Models 2011; 8: 5-11.
[34] Haas TL and Madri JA. Extracellular matrix-driven matrix metalloproteinase production in endothelial cells: implications for angiogenesis. Trends Cardiovasc Med 1999; 9: 70-77.
[35] Kessenbrock K, Plaks V and Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 2010; 141: 52-67.
[36] Dogrul AB, Colakoglu T, Kosemehmetoglu K, Birben E, Yaman E, Gedikoglu G and Abbasoglu O. Antiangiogenic response after 70% hepatectomy and its relationship with hepatic regeneration and angiogenesis in rats. Surgery 2010; 147: 288-294.
[37] Liekens S, De Clercq E and Neyts J. Angiogenesis: regulators and clinical applications. Biochem Pharmacol 2001; 61: 253-270.
[38] Nordin A SD, Weimann R, Studer P, Candinas D, Stroka DS. 355 Hypoxia inhibits liver regeneration after partial hepatectomy in mice. J Hepatol 2005; 42 Suppl 2: 131.
[39] Ronco MT, Francés D, de Luján Alvarez M, Quiroga A, Monti J, Parody JP, Pisani G, Carrillo MC and Carnovale CE. Vascular endothelial growth factor and nitric oxide in rat liver regeneration. Life Sci 2007; 81: 750-755.
[40] Higgins GM AR. Experimental pathology of the liver: restoration of the liver of white rat following partial surgical removal. Arch Patol 1931; 12: 186-202.
[41] Colakoglu T, Nursal TZ, Ezer A, Kayaselcuk F, Parlakgumus A, Belli S, Karakayali H and Haberal M. Effects of different doses of statins on liver regeneration through angiogenesis and possible relation between these effects and acute phase responses. Transplant Proc 2010; 42: 3823-3827.
[42] O'Reilly MS. Antiangiogenesis and vascular endothelial growth factor/vascular endothelial growth factor receptor targeting as part of a combined-modality approach to the treatment of cancer. Int J Radiat Oncol Biol Phys 2007; 69: S64-66.
[43] O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR and Folkman J. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 1997; 88: 277-285.
[44] Michalopoulos GK. Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas. Am J Pathol 2010; 176: 2-13.
[45] Taniguchi E, Sakisaka S, Matsuo K, Tanikawa K and Sata M. Expression and role of vascular endothelial growth factor in liver regeneration after partial hepatectomy in rats. J Histochem Cytochem 2001; 49: 121-130.
[46] Abdollahi A, Hahnfeldt P, Maercker C, Gröne HJ, Debus J, Ansorge W, Folkman J, Hlatky L and Huber PE. Endostatin's antiangiogenic signaling network. Mol Cell 2004; 13: 649-663.
[47] Abdollahi A, Hlatky L and Huber PE. Endostatin: the logic of antiangiogenic therapy. Drug Resist Updat 2005; 8: 59-74.
[48] Cattaneo MG, Pola S, Francescato P, Chillemi F and Vicentini LM. Human endostatin-derived synthetic peptides possess potent antiangiogenic properties in vitro and in vivo. Exp Cell Res 2003; 283: 230-236.
[49] Sasaki T, Larsson H, Tisi D, Claesson-Welsh L, Hohenester E and Timpl R. Endostatins derived from collagens XV and XVIII differ in structural and binding properties, tissue distribution and anti-angiogenic activity. J Mol Biol 2000; 301: 1179-1190.
[50] Sottile J. Regulation of angiogenesis by extracellular matrix. Biochim Biophys Acta 2004; 1654: 13-22.
[51] Nelson DL, Cox MM, Freeman WH; Company. Lehninger principles of biochemistry. Freeman WH and Company 2008.
[52] Delaney CE, Weagant BT and Addison CL. The inhibitory effects of endostatin on endothelial cells are modulated by extracellular matrix. Exp Cell Res 2006; 312: 2476-2489.
[53] Mongiat M, Sweeney SM, San Antonio JD, Fu J and Iozzo RV. Endorepellin, a novel inhibitor of angiogenesis derived from the C terminus of perlecan. J Biol Chem 2003; 278: 4238-4249.
[54] Shichiri M, Hirata Y. Antiangiogenesis signals by endostatin. FASEB J 2001; 15: 1044-1053.