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Vascular endothelial growth factor (VEGF) liver tissue concentration in delayed stages of rat liver regeneration upon interferon $\alpha 2b$ influence

Stężenie naczyniowo-śródbłonkowego czynnika wzrostowego (VEGF) w tkance wątrobowej w późniejszych etapach regeneracji wątroby szczura poddanej działaniu interferonu $\alpha 2b$

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Key words

vascular endothelial growth factor, rat liver regeneration, partial hepatectomy, interferon $\alpha 2b$

Słowa kluczowe

naczyniowo-śródbłonkowy czynnik wzrostowy, regeneracja wątroby szczura, częściowa hepatektomia, interferon $\alpha 2b$

Summary

Introduction. Vascular endothelial growth factor (VEGF) is postulated to play an important role in liver regeneration and interferon $\alpha 2b$ (IFN- $\alpha 2b$) is believed to inhibit this process. VEGF enhances proliferation of sinusoidal endothelial cells (SECc) *in vitro*, but its significance on liver regeneration *in vivo* is not well defined.

Aim. Investigation of the VEGF concentration in rat liver tissue in delayed stage of hepatic regeneration in baseline conditions and after IFN- $\alpha 2b$ administration.

Material and methods. The 45 three-months-old male Wistar rats were divided into three groups. The first group was injected subcutaneously with IFN- $\alpha 2b$ 24 h before and 24 h after partial hepatectomy (PH). The similar schedule was realized in the second group injected with 0.5 ml of 0.9% NaCl. The third group underwent sham-operation and was given two doses of IFN- $\alpha 2b$ with the 48 h interval. Rats were sacrificed in subgroups of five at 48, 72 and 96 h after surgery. The liver samples were obtained during surgery or autopsy. VEGF concentration was assayed in tissue homogenates with ELISA method.

Results. VEGF concentrations were not different before and after PH and IFN- $\alpha 2b$ had no significant influence on VEGF in analyzed time points.

Conclusions. VEGF pathway is not activated in rat liver between 48 and 96 h *post* PH and administration of IFN- $\alpha 2b$ has no impact on its tissue level neither in intact nor regenerating liver.

Streszczenie

Wstęp. Uważa się, że naczyniowo-śródbłonkowy czynnik wzrostowy (ang. *vascular endothelial growth factor* – VEGF) odgrywa istotną rolę w regeneracji wątroby, natomiast interferon $\alpha 2b$ (IFN- $\alpha 2b$) wywiera na ten proces wpływ hamujący. VEGF wzmacnia proliferację komórek śródbłonka zatok (ang. *sinusoidal endothelial cells* – SECc) *in vitro*, ale znaczenie tych oddziaływań w przebiegu regeneracji wątroby *in vivo* nie jest określone.

Cel pracy. Oznaczono stężenia VEGF w tkance wątrobowej szczura w późniejszej fazie regeneracji, zarówno podczas naturalnego przebiegu tego procesu, jak i po podaniu IFN- $\alpha 2b$.

Materiał i metody. 45 trzymiesięcznych samców szczurów rasy Wistar podzielono na trzy grupy. Pierwsza grupa otrzymała podskórnie IFN- $\alpha 2b$ 24 godziny przed częściową hepatektomią, a następnie 24 godziny po niej (ang. *partial hepatectomy* – PH). Ten sam schemat zastosowano w grupie drugiej, która otrzymała dwukrotnie podskórną iniekcję 0,5 ml 0,9% NaCl. Trzecia grupa otrzymała dwie dawki IFN- $\alpha 2b$ w odstępie 48 godzin, ale została poddana zabiegowi pozorowanemu. Szczury uśmiercano w podgrupach po pięć sztuk, kolejno w 48., 72. i 96. godzinie po przeprowadzonym

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zabiegu. Fragmenty tkanki wątrobowej pobierano w trakcie hepatektomii oraz podczas autopsji. Stężenia VEGF w homogenatach wątrób oznaczano metodą ELISA.

Wyniki. Stężenia VEGF przed i po PH nie różniły się, a podawany IFN- α 2b nie miał na niego istotnego wpływu w analizowanych punktach czasowych.

Wnioski. Szlak VEGF nie ulega aktywacji w wątrobie szczura pomiędzy 48. a 96. godziną po częściowej hepatektomii, a oddziaływanie IFN- α 2b nie ma wpływu na jego poziomy tkankowe ani w prawidłowej, ani w regenerującej wątrobie.

INTRODUCTION

Liver regeneration after the loss of effective hepatic mass is fundamental event in case of hepatic injury. Studies with hepatic resections showed that the regenerative response is proportional to the amount of liver removed (1, 2). The events triggered by partial loss of liver tissue involve mostly proliferation of hepatocytes, but also regeneration of all non-parenchymal mature cell populations. Hepatocyte proliferation starts in the periportal zone, continuing during next 36 to 48 hours towards pericentral areas of the lobules (3). The proliferation of non-parenchymal cells and the synthesis of new matrix are required for whole liver reconstitution (4, 5). Hepatic stellate cells (HSC), Kupffer cells and sinusoidal endothelial cells (SECs) enter cell cycle about 24 hours later than hepatocytes with a peak of DNA synthesis set at 48 h or later (6). Cellular sequential proliferations result in the formation of avascular liver tissue islands, with subsequent migration of the SECs into clusters of newly repopulated hepatocytes (7). Remodeling of regenerative liver architecture involves the formation of a complex network of sinusoids (8, 9). The stimuli to this process remain relatively unexplored.

Vascular endothelial growth factor (VEGF) is the best known angiogenic factor with documented growth-promoting effect on endothelial cells (10). VEGF is also a survival factor for SECs, as well as an inducer of their fenestrated phenotype important for microvascular permeability (11). There are several distinct isoforms of this homodimeric heparin-binding glycoprotein, which are the products of alternate splicing of the same gene (12, 13). VEGF binds to two receptor-type tyrosine kinases, Flt-1 (VEGF receptor-1) and KDR/Flk 1 (VEGF receptor-2), interacting with a family of co-receptors and membrane proteins (neurophilins), which do not contain a tyrosine kinase domain (10, 14).

Partial hepatectomy (PH), in which two-thirds (70%) of the liver is removed, is the widely accepted experimental rat model to study mechanisms of liver regeneration (15-19). However, the knowledge on regulation of sinusoidal net rebuilding during liver regeneration in partially hepatectomized rat is largely missing (20). SECs do not initiate DNA synthesis until 48 to 72 hours after resection, starting to divide approximately 96 hours *post* PH, with ongoing proliferation lasting at least 8 days following PH (6, 21). Assy et al. showed that serum levels of VEGF do not change significantly and remain on physiological levels following 70% PH (22). This finding encourages investigation of local behavior of VEGF concentrations.

The interferons (IFNs) are abundantly expressed cytokines, which show antiviral, immunomodulatory, growth-inhibitory and anti-fibrogenic activities (23-26). Moreover, interferon α (IFN α) is listed among angiogenesis inhibitors (27, 28). It is postulated, that activation of more than one signaling pathway is required for the generation of different effects of IFNs as no single signaling cascade is sufficient to reach any given biological end-point (29). To date, it has been not established if IFN administration has any impact on VEGF concentration within regenerating liver.

AIM

The purpose of this study was to investigate the hepatic concentration of VEGF in partially hepatectomized rats without pharmacological intervention and under influence of IFN α .

MATERIAL AND METHODS

Animal experiment

The 45 adult male Wistar rats (300-330 g) were maintained on rat chow and water under standard conditions with a 12-hour light-dark cycle. According to the study protocol they were divided into three groups, 15 of animals each. The first group (IFN/H) was injected subcutaneously with 0.5 ml IFN- α 2b (Intron A, Shering-Plough, 5 MU/100 ml 0.9% NaCl) and 24 hours later the 2/3 PH was conducted. After next 24 h the second IFN- α 2b dose was administered. The second group (IFN/O) was injected with the same doses of IFN- α 2b and was sham operated between the doses. Control rats (NaCl/H) underwent PH and received an identical volume (0.5 ml) of isotonic saline in the same time intervals in relation to PH. Injections and surgery (anesthesia: 50 mg/kg of ketamine given intraperitoneally) were carried out between 9.00-11.00 a.m. to minimize the influence of circadian variations. Rats were sacrificed in groups of five 48, 72 and 96 h after PH. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected by the *a priori* approval (no. 1/02; 19.02.2002) of the Local Ethics Commission for Animal Experiments of the Medical University of Silesia.

Samples of excised livers in IFN/H and NaCl/H groups were marked with "1", and liver samples in IFN/O group obtained during autopsy were marked with "2". The study design is shown in figure 1.

| | | | | |
|------------------------|---------------|----------------|---|-----|
| ⇓ →24h ⇨ PH (IFN/H/1) | 24h → ⇓ → 24h | ⇨ S (IFN/H/2) | } | 48h |
| ⇓ →24h ⇨ SO (IFN/O) | 24h → ⇓ → 24h | ⇨ S (IFN/O/2) | | |
| ⇓ →24h ⇨ PH (NaCl/H/1) | 24h → ⇓ → 24h | ⇨ S (NaCl/H/2) | | |
| ⇓ →24h ⇨ PH (IFN/H/1) | 24h → ⇓ → 48h | ⇨ S (IFN/H/2) | } | 72h |
| ⇓ →24h ⇨ SO (IFN/O) | 24h → ⇓ → 48h | ⇨ S (IFN/O/2) | | |
| ⇓ →24h ⇨ PH (NaCl/H/1) | 24h → ⇓ → 48h | ⇨ S (NaCl/H/2) | | |
| ⇓ →24h ⇨ PH (IFN/H/1) | 24h → ⇓ → 72h | ⇨ S (IFN/H/2) | } | 96h |
| ⇓ →24h ⇨ SO (IFN/O) | 24h → ⇓ → 72h | ⇨ S (IFN/O/2) | | |
| ⇓ →24h ⇨ PH (NaCl/H/1) | 24h → ⇓ → 72h | ⇨ S (NaCl/H/2) | | |

Fig. 1. The experiment schedule.

IFN/H – interferon α 2b-injected/hepatectomized animals; IFN/O – interferon α 2b-injected/sham-operated animals, NaCl/H – saline injected/hepatectomized animals; ⇓ – IFN/saline injections, PH – partial hepatectomy, SO – sham operation, S – sacrifice; “1” – tissue samples obtained during surgery; “2” – tissue samples obtained during autopsy; 48h, 72h, 96h – hours post surgery

Homogenization and total protein concentration

Weighed samples of rat liver (100 mg) were homogenized using a PRO 200 homogenizer (PRO Scientific Inc, USA) at 10 000 RPM in nine volumes of phosphate-buffered saline solution (PBS without Ca and Mg, BIOMED, Poland) containing 0.5% Triton X-100 (Sigma-Aldrich, USA). Then homogenates were centrifuged at 12 000 RPM for 15 minutes at 4°C, and supernatants were divided into appropriate portions and frozen at – 80°C until required for further surveys.

The total protein concentration was determined using pyrogallol-red method using a set of reagents for direct colorimetric measurements of total protein (Sentinel Diagnostics, Italy). Readings were taken at 600 nm wavelength at 37°C using Technicon RA-XT biochemical analyzer (Technicon Instruments Corporation, USA).

VEGF concentration

The VEGF concentration was assayed in rat liver homogenates by ELISA method according to the RayBio Rat VEGF ELISA Kit (RayBiotech, Inc., USA) assay kit instructions in duplicates. Absorbance readings were obtained with ELISA PowerWave XS (BioTek, USA) at 450 nm wavelength and calibrated according to standard curve in pg/ml. The results obtained were calculated for 1 mg of liver homogenate total protein content.

Statistics

Statistical analysis was carried out using STATISTICA 10.0 PL software. Descriptive statistics were calculated and the data were tested for normality of distribution and homogeneity of variance. For independent sample differences between groups the analysis of variance and

post hoc tests were done while for dependent variables the t tests for dependent samples were used. P < 0.05 was considered as statistically significant.

RESULTS

Experiment schedule allows to make several different observations: during PH we gained the normal liver tissue samples (NaCl/H/1) and liver tissue exposed to one dose of IFN- α 2b administered 24 h before (IFN/H/1). Autopsy conducted in consecutive time points after sham operations: 48, 72 and 96 h provided the liver treated with two doses of IFN- α 2b administered in 48-h interval (IFN/O/2). In the group treated with IFN- α 2b and partially hepatectomized between two doses, during autopsy we obtained the regenerating liver tissue exposed to IFN- α 2b (IFN/H/2), while in the group injected with saline only we obtained liver tissues in later stages of regeneration induced by PH (NaCl/H/2). Concentrations of VEGF in liver tissue samples of all study groups are gathered in the table 1.

In our experiment all samples marked NaCl/H/1 taken together represent the normal liver and all denoted IFN/H/1 are liver samples in 24h after one dose of IFN α 2b. Comparison of VEGF concentrations between these two groups shown no important difference. In sham-operated and twice injected of IFN- α 2b rats there were no differences in VEGF concentrations in livers obtained in consecutive time points (48 vs 72 vs 96 h) after surgery (tab. 1).

Later stages of PH-induced liver regeneration were observed up to 96 hrs after surgery. VEGF concentrations in normal liver samples obtained during PH compared with those estimated in regenerating livers

Table 1. VEGF concentrations in rat livers in all study groups at time points according to experiment schedule.

| VEGF [pg/mg protein]; mean \pm SD | | | | | | | |
|-------------------------------------|----------------|-----------------|-------------|-----------------|-----------------|-------------|-----------------|
| Time after surgery | NaCl/H/1 | NaCl/H/2 | p 1 vs 2 | IFN/H/1 | IFN/H/2 | p 1 vs 2 | IFN/O |
| 48 h | 303 \pm 56.2 | 137 \pm 135.2 | NS | 297 \pm 102.0 | 314 \pm 119.9 | NS | 358 \pm 96.4 |
| 72 h | 221 \pm 41.0 | 218 \pm 89.7 | NS | 366 \pm 126.9 | 315 \pm 2.2 | NS | 184 \pm 109.4 |
| 96 h | 136 \pm 4.8 | 127 \pm 11.9 | NS | 196 \pm 20.9 | 143 \pm 82.0 | NS | 133 \pm 40.4 |
| p 48 h vs 72 h vs 96 h | – | NS | – | – | NS | – | NS |

NaCl/H/1 – normal liver; IFN/H/1 – liver exposed to one dose of IFN- α 2b; IFN/O – sham operated animals, liver exposed to two doses of IFN- α 2b; IFN/H/2 – regenerating liver, rats exposed to two doses of IFN- α 2b; NaCl/H/2 – regenerating liver; “1” – tissue samples obtained during surgery; “2” – tissue samples obtained during autopsy

(NaCl/H/1 vs NaCl/H/2) in consecutive time points shown no important differences. Similar results we obtained comparing VEGF concentrations in liver exposed to one dose of IFN- α 2b with estimations in regenerating liver tissue in rats treated with two doses of this cytokine (IFN/H/1 vs IFN/H/2) (tab. 1). In fact, comparing VEGF concentrations in livers obtained during autopsy in all study groups in given time points with those estimated in normal liver we could not find important differences (fig. 2). Next, the relationship between VEGF concentrations in liver exposed to one dose of IFN- α 2b and liver tissue, both normal and regenerating, in rats treated with two doses, also shown no statistical importance (fig. 3).

DISCUSSION

Angiogenesis is not only an integral part of tumorigenesis, but also plays a role in chronic liver disease being crucial step in hepatic regeneration and repair (30-33). Partial resection of the rat liver provides a model to investigate the role of structurally and functionally intact SECs in supporting liver regeneration (34). It is postulated that VEGF promotes liver regeneration by regulating the proliferation of SECs and reconstruction of liver sinusoids (20, 35). VEGF was shown to be expressed in hepatocytes, while its receptors in sinusoidal endothelial cells, what suggest possible communication between proliferating hepatocytes and SECs via VEGF induced pathway (36). In regenerating liver SECs population mostly consists of proliferating endothelial cells, but bone-marrow-derived cells sensitive to VEGF and transforming into endothelial cells were also identified (10, 37).

Among VEGF isoforms, VEGF₁₈₉ and VEGF₂₀₆ are highly basic and bind to heparin with high affinity, VEGF₁₆₅ has intermediary properties, its significant fraction remains bound to the cell surface and extracellular matrix (ECM), while VEGF₁₂₁ is an acidic freely diffusible polypeptide that does not bind to heparin (10, 27). The VEGF₁₆₅ has the highest biological activity and is the most representative product of VEGF gene in both the normal and activated cells (12). In this study we measured VEGF concentration in liver homogenates, without distinguishing between different isoforms, therefore, our results mostly refer to ECM and cell surface bound molecules.

According to experimental evidence SECs start DNA synthesis after 48 h or later following PH and endothelium proliferation is beginning since the 4th day and plateaus until day 8, concomitantly with increased expression of the receptors *flt-1* and *KDR/flk-1* (6, 34, 38). In our study hepatic concentrations of VEGF were measured in 48, 72 and 96 h after PH and in this period of time we did not find any significant elevations of VEGF concentrations as compared with baseline values. There are several probable explanations for this unexpected finding. First explanation is that angiogenesis takes place despite lack of significant activation of VEGF production and release. It is known that blood vessel formation is an orchestrated process involving many other factors, such as members of the platelet-derived growth factor or transforming growth factor- β families as well and other gene products – ranging from transcription factors to members of the Notch family (39). Shergill et al. showed that lack of VEGF and NO-dependent angiogenesis does not impair liver regeneration in PH mice model with heterozygous deficiency of the VEGF receptor (40). This finding implies that absence of the canonical vascular pathway is not necessary for undisturbed liver regeneration. The drawback of our study is the absence of pathomorphological monitoring of angiogenesis within regenerating liver.

The second explanation could be not appropriate timing of VEGF measurement. There is evidence that

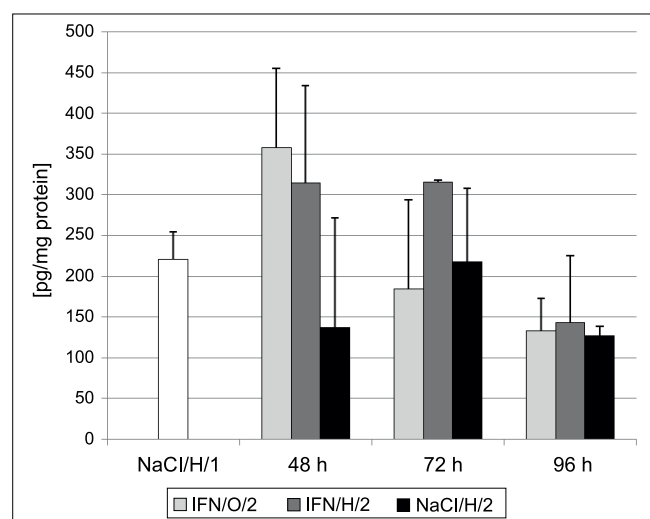


Fig. 2. Liver VEGF concentrations in different experimental models. NaCl/H/1 – normal liver; IFN/O/2 – sham operated animals, liver exposed to two doses of IFN- α 2b; IFN/H/2 – regenerating liver, rats exposed to two doses of IFN- α 2b; NaCl/H/2 – regenerating liver; “1” – tissue samples obtained during surgery; “2” – tissue samples obtained during autopsy; 48 h, 72 h, 96 h – hours post surgery

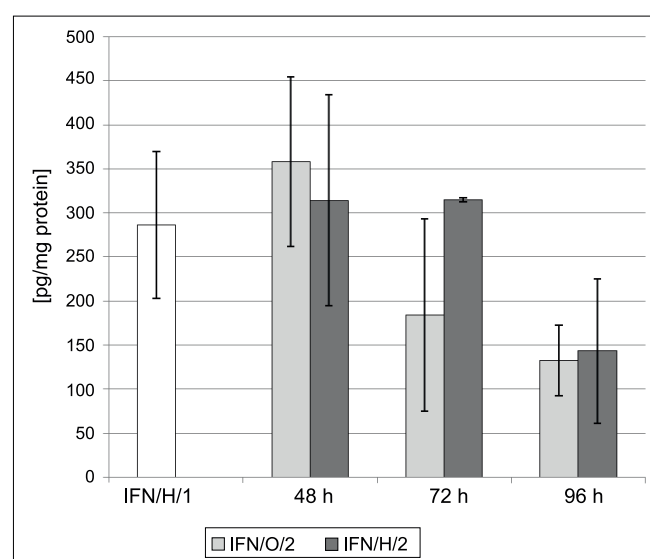


Fig. 3. Liver VEGF concentrations in hepatectomized and not hepatectomized rats exposed to IFN- α 2b. IFN/H/1 – liver exposed to one dose of IFN- α 2b; IFN/O/2 – sham operated animals, liver exposed to two doses of IFN- α 2b; IFN/H/2 – regenerating liver, rats exposed to two doses of IFN- α 2b; 48 h, 72 h, 96 h – hours post surgery

VEGF expression in rat liver is increased in periportal hepatocytes 48-72 h after PH and neutralization of this growth factor significantly inhibited proliferation activity of SECs at 48 and 96 h (41). However, in other study an increased maximal hepatic VEGF-mRNA expression in hepatectomized rats was found at 72 h after the surgery, and mRNA expression of VEGF receptors between 72 and 168 h (42). As it was stated SECs division starts about 96 h post PH (6, 21), probably partly as a result of VEGF molecules interaction with their receptors. Therefore, we cannot exclude that significant amounts of VEGF might occur later than observation time points in our protocol. Moreover, our study was focused on VEGF concentrations measured in tissue homogenates, which could reflect the average relative concentrations of the finally synthesized molecules, but those connected with cell surface receptors could not be detected with ELISA method.

In this study we compared, but did not find important differences in VEGF tissue levels in regenerating rat liver measured in the setting with and without influence of IFN- α 2b. In rat model the administration of IFN α after PH is responsible for inhibition of hepatic

regeneration, especially affecting the DNA and total protein synthesis by hepatocytes (43-45). These observations refer mainly to the first 24 h after PH. Lanford et al. analyzing the transcriptional response of chimpanzees liver tissue and peripheral blood mononuclear cells to IFN α using microarrays concluded that the signal transduction-transcriptional pathway affected by IFN rapidly expires and time required to regain responsiveness approximates 24 h (46). In our study the second dose of IFN- α 2b was administered 24 h after surgery, thus its influence probably covered the period of 48-72 h after PH. Therefore, we can state that in this period of time of the experiment IFN- α 2b had no suppressive effect on VEGF in the stage of non-activation of synthesis.

CONCLUSIONS

Liver may regenerate without significant activation of VEGF as its hepatic tissue concentration is not increased in delayed stage of liver regeneration and IFN- α 2b administration has no impact on VEGF tissue levels either in intact or regenerating liver.

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