Predictive Value of Growth Factors and Interleukins for Future Liver Remnant Volume and Colorectal Liver Metastasis Volume Growth Following Portal Vein Embolization and Autologous Stem Cell Application

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Abstract. *Background: Liver metastases occur in 60-80% of patients with colorectal carcinoma. The only potentially curative method is surgical resection, with an operability of 20-25%. The main reason for such low resectability is insufficient future liver remnant volume (FLRV). Portal vein embolization (PVE) alone is associated with failure in up to 40% of patients. A new method that could lead to acceleration of FRLV growth appears to be combination of PVE and application of hematopoietic stem cells (HSCs). The aim of our study was to evaluate the importance of growth factors and interleukins for FLRV growth after PVE and HSC application and also their possible effect on growth of colorectal liver metastases. Patients and Methods: From June 2010 to July 2014, PVE was combined with application of adult HSCs in 16 primarily inoperable patients with colorectal liver metastases. We determined the serum levels of growth factors [hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF-1), insulin-like growth factor binging protein 3 (IGF-BP3), epidermal growth factor (EGF), transforming growth factor (TGFα), tumor necrosis factor (TNF)] and interleukins (IL2, -6, -8 and -10) at given time intervals by immunoanalytic methods. The growth of FLRV was evaluated by multidetector computed tomography at intervals of 1 week until sufficient growth of FLRV. Results: We were able to perform radical surgery in 13 primarily inoperable patients (81.4%). The average FLRV growth was 23.1% (range=21.9-38.6%); from an initial FLRV of 30.5% (range=20.6-39%) to 40.1% (range=29-48%) before resection. The combination of levels of EGF, HGF, VEGF, IGF, TGFα and IL2,-6,-8 appears to be crucial for predicting operability. IL8 was statistically significant for the growth of colorectal liver metastases, and TGFα, IL2, and IL8 are important for a longer disease-free interval.*

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Key Words: Portal vein embolization, autologous stem cells, growth factors, interleukins.

The discovery of human stem cells in 1998 was associated with great hope regarding the potential for replacement of individual tissue and organ functions (1). Lately, many clinical institutions worldwide have endeavored to use the regenerative and reparative abilities of stem cells for the treatment of various diseases. The application of stem cells promotes regeneration of tissues damaged by degenerative or lifestyle diseases *via* various mechanisms. The possibility of improving the regenerative abilities of the liver parenchyma has been repeatedly demonstrated by several independent studies (2-4) namely in congenital diseases.

Stem cells can be divided into embryonic and adult stem cells, that can be obtained in several ways. In our study, we used adult hematopoietic stem cells (HSC) which carry the surface markers CD133 (prominin 1) and CD34. We acquired these cells by peripheral blood apheresis following stimulation with granulocyte colony-stimulating factor (G-CSF).

Given that colorectal carcinoma is the second most frequent malignant disease worldwide, it represents a serious socioeconomic problem. Currently, its incidence is shifting towards younger age groups. Colorectal liver metastases (CLM) are diagnosed in 60-80% patients suffering from colorectal carcinoma. Synchronous CLM occur in approximately 15-40% of patients and metachronous metastases in 60-70% of patients (5). Multimodal therapy is based on radical surgical resection of CLM, leading to a 5-year patient survival of 38-56% (6, 7). Nevertheless, at present, primary resecability of CLM still remains in the range of 20-25% (8). The main problem of low CLM resectability is the insufficient future liver remnant volume (FLRV).

There are several possibilities to increase FLRV. Portal vein embolization (PVE) remains the gold standard for increasing FLRV. As soon as the volume of the contralateral liver lobe increases, liver resection is performed. The interval necessary for FLRV hypertrophy usually ranges between 2 and 8 weeks (9). However, despite successfully technically performed PVE, hypertrophy does not occur in a number of patients (approximately 20-40%). There is also danger of CLM progression during the time required for FLRV hypertrophy. The application of HSCs immediately after PVE provides the possibility of increasing the regenerative speed of liver parenchyma and improving its function, especially in patients with previous impairment of the parenchyma, for example due to prolonged chemotherapy, steatosis, or fibrosis (10). Growth factors and interleukins are undoubtedly important for the growth of FLRV. The question remains, though, to what extent these factors also affect the progression of CLM.

The aim of our study was to evaluate the predictive value of growth factors and interleukins for the growth of FLRV or possible progression of CLM following PVE and application of HSCs.

Patients and Methods

We retrospectively evaluated a group of 16 patients with CLM primarily inoperable due to insufficient FLRV. We used the combination of PVE and application of HSCs for support of FLRV growth. We first used this method in clinical practice in July 2010. Before inclusion of the first patient, the method was approved by the Ethics Committee of our University Hospital (No. 326/2011). Twelve men and four women were included in this study. The average age of the women was 59.7 (53-67) years and that of the men was 65.7 (51-75) years. The study included patients with primarily inoperable CLM due to insufficient FLRV. Patients with extrahepatic metastases were excluded. The average number of metastases was 4.5 (range=1-14). Insufficient FLRV was considered to be a volume less than 25% in the anticipated healthy parenchyma and 30-35% in patients where the liver parenchyma was damaged by a primary disease (steatosis, cirrhosis) or previous prolonged chemotherapy. This involved more than eight cycles in 12 patients. Before patient inclusion in this study, we performed initial computed tomographic (CT) volumometry of the liver and determined FLRV (SomatomDefinition; Siemens, Erlangen, Germany). The average initial FLRV was 30.5% (range=21.9-38.6%). Liver parenchymal function was evaluated according to clinical and laboratory parameters as well as by using the indocyanine green (ICG) retention test (Limon; PulsionMedical Systems AG, Munich, Germany). Alteration of the ICG test was seen in nine patients (Table I). All patients were properly informed regarding the proposed treatment process and were warned about possible risks (particularly the possibility of CLM progression) and they signed an informed consent form. The procedure was determined by a multidisciplinary team (surgeon, radiologist and oncologist).

In all patients, serum levels of growth factors were determined before surgery, after PVE and the application of HSC and at 1-week intervals. The growth factors hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF1), insulin-like growth factor-binding protein 3 (IGFBP3), epidermal growth factor (EGF), transforming growth factor alpha (TGFα) and tumor necrosis factor alpha (TNFα) were evaluated. We also determined inflammatory cytokines/interleukins (IL)-2, -6, -8, -10 and tumor necrosis factor alpha (TNFα) in the same blood samples. We monitored the relationship of serum levels of these factors on the growth of FLRV and also on the volume of CLMs over time.

Table I. *ICG test results (normal value, R15<10%).*

ICG test alteration	Number of patients	Mean R15 (%)
Yes	7	14.8
No	9	3.4

ICG: Indocyanine green.

Serum levels of growth factors IGF1 and IGFBP3 were determined by immunoradiometric assay (IRMA) (Beckman Coulter, USA, and DiaSource, Belgium, respectively). The quantification of plasma levels of HGF, EGF, TGFα, IL10, IL2, IL6, IL8, TNFα and VEGF was based on magnetic bead multiplex immunoassays (Millipore, Billerica, MA, USA) and MagPix instrument (Luminex Corporation, Austin, TX, USA). The MILLIPLEX Human Cytokine/Chemokine Magnetic Bead Panel was used to measure EGF, TGFα, IL10, IL2, IL6, IL8, TNFα and VEGF and the MILLIPLEX Human Adipokine Magnetic Bead Panel 2 was used to quantify HGF.

The whole process of PVE and the application of HSC involved several steps. Firstly, we performed PVE *via* the transparietal route using a mixture of Histoacryl (BBraun, Tuttlingen, Germany):Lipiodol (Guerbet,Rennes, France) 1:10. Next, we stimulated HSC release from bone marrow using G-CSF (filgrastim; Neupogen, AmgenEurope B.V, Breda, Holland) at a dose of 10 μg/kg/day. Starting on the fourth day of stimulation, the level of $CD34^+$ and $CD133^+$ HSCs was monitored by means of flow cytometry. If the level was sufficient, apheresis was performed using a Cobe Spectra continuous blood cell separator (BCT, Lakewood, CO, USA), using a program for the collection of mononuclear leukocytes (MNC program, software version 6.1 (Baxter, Deerfield, IL, USA). Two volumes of the patient's blood were processed and a citrate and citric acid-based solution (ACD-A, Baxter, Deerfield, IL, USA) at a ratio of 1:12 to 1:14 to full blood was used as anticoagulant. The volume of the obtained product was 200 ml (range=187-232 ml). We then made an alternating incision in the right lower abdomen under general anesthesia. We cannulated one of the branches of the *vena ileocolica*. Afterwards, the catheter was selectively placed in the non-embolized portal vein branch under sciascopic control and the product was applied as a slow infusion into the contralateral liver lobe. Growth of the contralateral liver lobe was monitored by means of multidetector CT (MDCT) volumometry at 1-week intervals following the application of HSCs. Resection of the liver was performed as soon as the growth of the contralateral liver lobe volume was sufficient.

Statistical analysis was performed using the SAS (SAS Institute Inc., Cary, NC, USA) and STATISTICA (StatSoft Inc., Tulsa, OK, USA) software. Basic statistical data, such as the average, standard deviation, dispersion, median, interquartile range, minimum and maximum were derived for the measured parameters in the whole

Table II. *Cut-offs for serum parameters indicative of successful liver resection.*

Molecule	Name	Cut-off	*p*-Value
EGF	Epidermal growth factor	<80 pg/ml	0.0455
HGF	Hepatocyte growth factor	>310 pg/ml	0.0455
VEGF	Vascular endothelial growth factor	<200 pg/ml	0.0909
IGF	Insulin-like growth factor	>90 ng/ml	0.0909
IL6	Interleukin 6	>35 pg/ml	0.0909
IL8	Interleukin 8	>50 pg/ml	0.0909
IL2	Interleukin 2	<3.21 pg/ml	0.05454
TGFα	Transforming growth factor α	<3.21 pg/ml	0.05454

group, as well as in individual groups and subgroups. Spearman's correlation coefficient was used to determine the dependencies of the studied parameters given the non-Gaussian division of these variables. The difference in investigated parameters between the groups was tested using the two-sided Wilcoxon test. For selected endpoints, the relationship between the investigated markers or their changes and the endpoint was expressed as specificity, sensitivity and risk (odds ratio; OR). These differences were analyzed using Fisher´s exact test. The evolution of the studied parameters over time was tested using the Wilcoxon test for paired samples as well as a paired-sample median test, and where more time points were available, we tested the evolution using Friedman ANOVA test or parametric repeated ANOVA test. The calculation of overall (OS), disease-specific (DFI) and progression-free survival (PFS) was performed using the Kaplan–Meier test. The difference in survival between the studied groups was tested using the Wilcoxon and log-rank tests. For the tested markers, the cut-off was set at the level of the median (patients were divided into two groups containing the same number of patients). The influence of individual factors on OS and PFS was modeled using the Cox regression model. Given the small number of patients and the great number of tested variables, only univariate analysis was performed. The statistical significance was set at a level of alpha=10%.

Results

There were no serious complications of PVE and HSC application in any of the patients. In one patient (6.3%), there was leakage of the embolization material into the left segments of the liver, with no impairment of liver function. After leukapheresis, mild citrate toxicity occurred in some patients. A volume of 10,000 ml of blood (range=6,000-12,000 ml) per patient was processed and collection of HSC product lasted 138 min (range=129-154). The apheresis product contained 10.9×10^7 (range=12.96-23.30$\times10^7$) $CD34^+$ cells and 5.83×10^7 (range=11.19-19.10$\times10^7$) $CD133^+$ cells. The minimum number of $CD34^+$ and $CD133^+$ cells in the collected product should be over 2×10^7 and 1×10^7, respectively. The required dose of HSC was met in 100% of collections.

The 30-day mortality rate was 0% and morbidity was 12.5%. Pneumonia occurred in one patient and confusion lasting several days and requiring psychiatric intervention was observed in another. The average total liver volume (TLV) was 1,734.5 (range=866-2448) cm^3 before the procedure and 1,724.5 (range=977-2406) cm^3 after it. Before PVE with HSC application, the FLRV was 535.9 (range=179-880) cm^3 *i.e.* 30.5% (20.6-39%) of the TLV. Before resection, the FLRV had increased to 689.2 (range=421-862) cm^3, corresponding to 40.1% (range=29-48%) of the TLV. The total increase of parenchymal mass in the non-embolized part of the liver was 23.1% (range=0-47%). The average time required for sufficient hypertrophy was 20 (range=14-25) days.

The volume of CLMs was 76.6 (range=6-465) cm^3 before surgery. During the interval between PVE with application of HSC and liver resection, the average CLM volume increased to 129.8 (range=5-832) cm^3; this was a significant increase of 77.6%. The growth of CLM was independent of the previous chemotherapy.

Resection of the liver was performed in 13 (81.4%) patients. The most frequent procedure involved was extended right hepatectomy (n=7). Six patients underwent right hepatectomy. It was not possible to perform surgery in three patients (18.6%). In two patients, the reason was progression of CLM; in the third patient, despite sufficient FLRV growth, resection was not technically possible due to severe intra-abdominal adhesions following previous surgeries. For these patients, we continued with adjuvant oncological treatment.

We found some interesting correlations between serum levels of evaluated markers, CLM and FLRV growth. From the point of view of CLM growth, IL8 ($p<0.0083$) appeared to be a significant serum prognostic marker, whereby a value exceeding or equal to 6.3 pg/ml was protective and a value less than 6.3 pg/ml always signified a risk of progression of metastasis volume (Figure 1). Another important prognostic marker was TGFα, whereby serum levels before PVE with the application of HSC of less than 3.9 pg/ml characterized long PFI and on the contrary, levels ≥3.9 pg/ml were indicative of CLM recurrence within 6 months after liver resection (Figure 2). A similar cut-off was found in the case of IL8 and IL2, whereby early recurrence of CLM after

radical liver resection was seen at serum levels ≥26 pg/ml (Figure 3) and ≥3.2 pg/ml, respectively.

The serum IGF level was statistically significantly indicative of FLRV growth of over 35%. The serum level of IGF remained more or less stable in patients with FLRV growth of good quality from PVE with the application of HSC to resection. In contrast, in cases where liver hypertrophy was not sufficient, a significant decrease of IGF ($p<0.0364$) was recorded in the course of the first 2 weeks (Figure 4).

We also evaluated the significance of serum marker levels before liver resection for success of this procedure. We found that cut-offs significant for serum levels indicative of successful liver resection were below 80 pg/ml for EGF and above or equal than 310 pg/ml for HGF (both with $p<0.0455$), below 200 pg/ml for VEGF, above or equal than 90 ng/ml for IGF, above or equal than 35 pg/ml for IL6, above or equal than 50 pg/ml for IL8 ($p<0.0909$ in all cases), and below 3.21 pg/ml for IL2 and TGFα ($p<0.0545$) (Table II).

The dynamics of certain factors following PVE with the applications of HSC were interesting. We observed elevation of serum levels of IGF, IGFBP3, HGF and IL8 in the first week after HSC application, with a subsequent decrease in the weeks before liver surgery. It was also shown that after hepatic resection, the liver parenchyma no longer reacted in the form of greater changes of growth factor or IL levels (Figure 5), but rather liver parenchyma processes appeared to continue in the set process of liver regeneration.

The median patient OS was 2.1 years. Eleven patients (68.75%) are still alive. Four patients had died of tumor progression and one patient died of cholangiogenic sepsis. The median follow-up is now 9 (range=1-45 months). There has been no disease progression in 43.8% of patients (Figure 6), and the median PFI is 0.77 years.

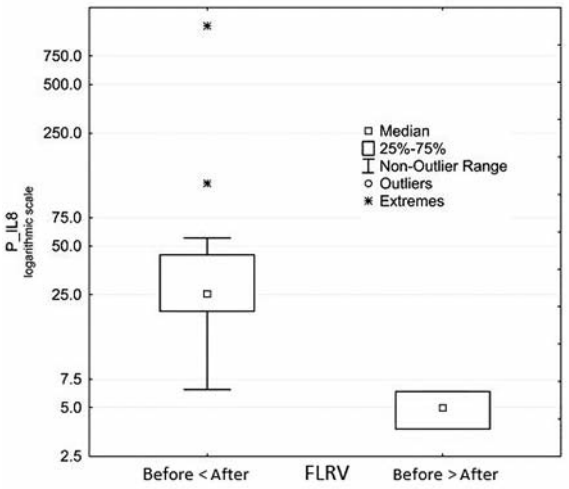

Figure 1. *Serum interleukin 8 (IL8) level as a prognostic marker of colorectal liver metastases (CLM) growth.*

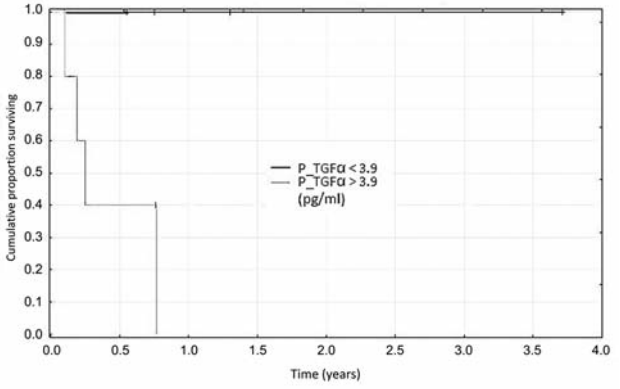

Figure 2. *Serum transforming growth factor-α (TGFα) levels before portal vein embolization (PVE) as a prognostic marker for progression free interval (PFI).*

Discussion

The history of liver regeneration by influencing blood flow in the portal bloodstream goes back to 1920. The first experiments were carried out on rabbits, where after portal vein ligation, atrophy was observed on the same side and corresponding hypertrophy was observed on the contralateral side (11). Portal vein ligation in a human as part of a two-stage liver resection was first published in 1961 (12). Since then, many open routes of portal vein occlusion have been tested and published. The first PVE was performed in 1982 through the ileocolic access (13) and for the first time was published by Makuuchi *et al.* in 1990 (14).

PVE is a successful method for FLRV growth in up to 60% of cases on average. However, in many patients, CLM progression occurs, probably as a consequence of the relatively long interval required for liver hypertrophy. Therefore, other methods that can shorten the interval required for the growth of FLRV to a minimum are beingsought. One such method is associating liver partition and portal vein ligation for staged hepatectomy (ALPPS), which was performed as a surgical novelty by Lang *et al.* in 2011 (15). Further cases and methodologies were subsequently published by De Santibañes *et al.* in 2012 (16)/. Despite the excellent hypertrophy of liver parenchyma within an interval of 1 week, this procedure is associated with high mortality (12-22%). This mortality is not acceptable for liver resection under our circumstances. We are inclined to agree with the opinion of the Japanese and American schools of thought that current trends should focus on minimizing perioperative mortality (17, 18). This is why, like many other institutions (19, 20), we searched for a different method, one that would be highly effective for rapid FLRV growth and have a low mortality and morbidity rate.

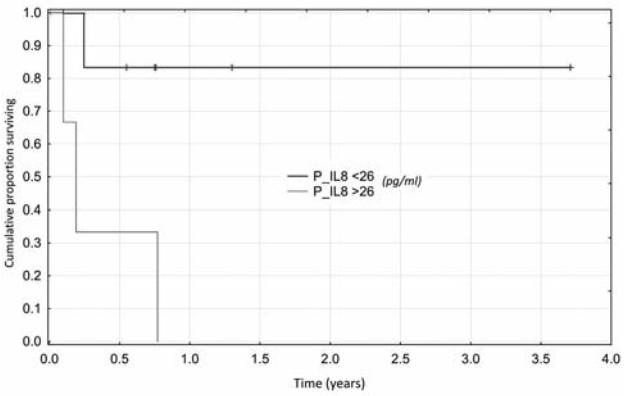

Figure 3. *Serum interleukin 8 (IL8) levels as a prognostic marker of progression-free survival.*

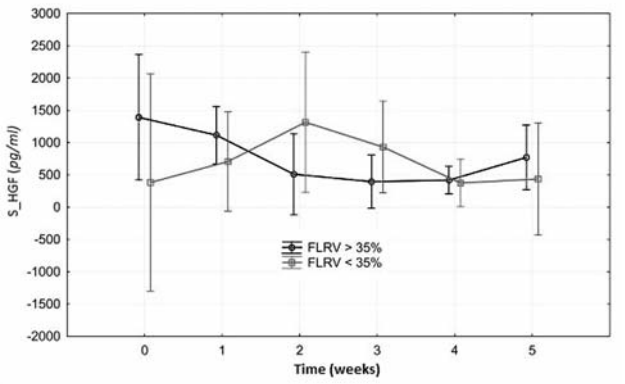

Figure 5. *Changes in Hepatocyte growth factor (HGF) levels over time.*

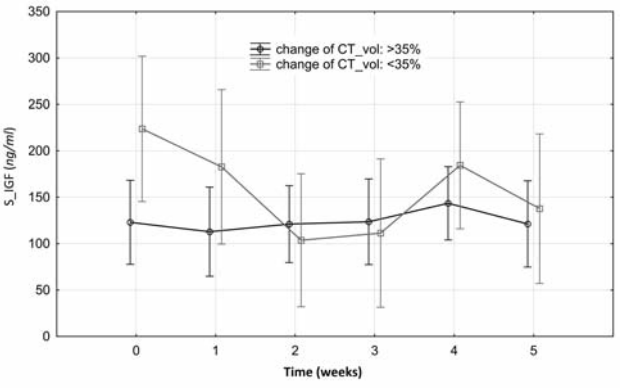

Figure 4. *Pattern of S-insulin-like growth factor (S-IGF) levels over time in patients with and without future liver remnant volume (FLRV) >35%.*

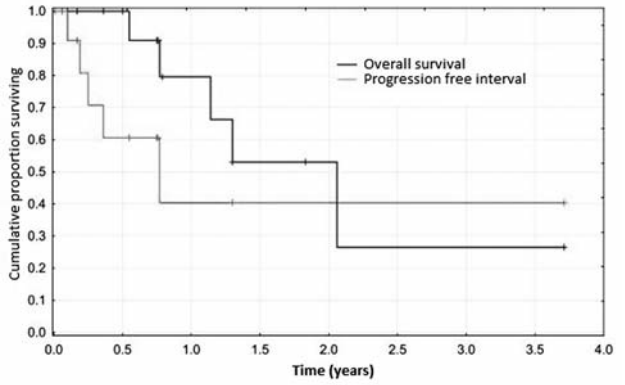

Figure 6. *Survival analysis of the whole patient cohort.*

Stem cells appear to be a new auspicious option for replacing the damaged functions of individual organs. Nowadays, they are especially used in the field of regenerative medicine to improve the function of the heart, liver, brain, spinal cord *etc*. Pluripotent cells which are able to differentiate into all cell lines could have the highest potential. Embryonic and induced pluripotent stem cells can be considered to be such cells. Unfortunately, both are associated with a high risk of the occurrence of teratomas. Therefore, clinical application requires other, more highly differentiated cells, for example hematopoietic progenitor and mesenchymal cells obtained from bone marrow, mobilized peripheral blood or adipose tissue (21). These cells are also termed adult stem cells. Despite the fact that some of these cells directly transdifferentiate, this transformation is rare and not overly significant and it cannot directly explain the improvement of organ function. The effect of HSCs is not conditional on their implantation within the liver parenchyma but a minimum proportion (3-5%) of HSCs transdifferentiate in the liver parenchyma. The major function seems to be their impact on 'sleeping' stem cells (oval cells) and their significant paracrine effects (22) on growth factors, ILs, bioactive lipids and extracellular microvesicles, which are released from the cells and have trophic, antiapoptotic and angiopoietic effects (23).

The application of stems cells derived from bone marrow in advanced diffuse diseases of the liver parenchyma, including cirrhosis, has also been repeatedly published (24). That is why our group focused on patients following prior prolonged chemotherapy (more than six cycles). Nine patients in our group (56.25%) had undergone prolonged chemotherapy prior to liver resection. We then used our method in patients with elevation of liver tests and those with poor functional status of the liver parenchyma.

Progression of the growth of CLM volume was seen in many patients after PVE and the application of HSCs. The

same phenomenon was noted in patients in whom we used only PVE without any application of HSCs (unpublished data). Thus, growth of CLM volume seems to be independent of the application of HSCs. We found no connection between neoadjuvant oncological treatment and growth of CLMs after PVE with the application of HSCs either. Unfortunately, progression of CLMs was the principal reason why it was impossible to perform radical liver surgery. Similar experience was published by Simoneau *et al*. (25). It has been shown that metastases of 1 cm in diameter and more are nourished better by arterial blood (26). In our opinion, progression of CLM size is associated with the hepatic arterial buffer response. In accordance with other studies (27), the volume of blood flow in the embolized lobe is renewed within approximately 14 days after PVE. There is an increase in the flow rate through the hepatic artery, as well as in the area of the arterial sinusoidal network, where it is possible to observe sinusoidal dilatation during microscopic examination. Blood flow restoration to the level before PVE leads to the arterialization of the whole embolized lobe.

HGF, the serum level of which increases during liver regeneration and this increased level can be recorded as early as a few hours from onset, is the principal initiator of liver regeneration, acting directly on hepatocytes. TNF is produced by liver macrophages (Kupffer cells) and has various functions. It is most often produced as a response to bacterial or other infections, tumor growth *etc*. TNF production is promoted by ILs. TNF also induces *e.g*. production of the EGF receptor. If its level drops, liver regeneration slows-down significantly. EGF is produced by Brunner cells (duodenum) and is a strong mitogen for hepatocytes. IGF is a protein that binds to plasma proteins. It stimulates tyrosine kinase and thus stimulates cell differentiation and division. It is mainly produced in the liver. VEGF stimulates angiogenesis and higher levels are associated with insufficient oxygen supply to tissues. It contributes to the proliferation of endothelial cells and to increased vascular permeability.

Interleukins are proteins that play a significant role in the process of inflammation. They are produced not only by leukocytes, but also by a great number of other cells (epithelial and dendritic cells, endothelium *etc*.). They have a very important role in liver regeneration.

Literature focusing on growth factors and ILs during liver regeneration mostly includes articles regarding liver resection or liver trauma (28-31). Our study deals with the significance of these factors from the aspect of FLRV, CLM growth in patients following PVE with the application of HSCs. In this aspect, this is the first such publication in the current literature. We have demonstrated that changes in the serum levels of certain factors occur following PVE with the application of HSCs. This was evident especially in the case of IGF, IGFBP3, HGF and IL8 levels. These changes corresponded to changes observed following liver resection or liver trauma. IGF is important for the growth of FLRV, whereby a stable level of this marker during the period of hypertrophy appears to be favorable. In contrast, during changes in IGF levels, no significant hypertrophy occurred in our group of patients. Serum levels of IL8 before PVE with HSC application were significantly related to the changes in CLM volume; higher levels appeared to be protective. Therefore, serum values of these markers could be used in the future to predict the development of FLRV and the risk of CLM growth, as well as to determine patient prognosis. This could be important from the aspect of patient follow-up and naturally from the aspect of subsequent adjuvant oncological treatment.

Conclusion

Our study has certain limitations, principally given the small number of patients and the short period of follow-up. The limitations are due to the uniqueness of the group of patients and the strict indication criteria. Nonetheless, even these preliminary results represent encouraging information regarding this newly-implemented method, as well as important data on the significance of certain growth factors and ILs for liver regeneration and CLM growth following PVE with the application of HSCs.

Acknowledgements

Supported by the Ministry of Health, Czech Republic - Conceptual Development of Research Organization (Faculty Hospital Pilsen - FNPl, 00669806, NS 96-44) and Research Project of Charles University in Prague P 36.

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Received December 29, 2015
Revised February 22, 2016
Accepted March 2, 2016