Angiogenesis in Synchronous and Metachronous Colorectal Liver Metastases

The Liver as a Permissive Soil

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Objective: Resection of a primary colorectal carcinoma (CRC) can be accompanied by rapid outgrowth of liver metastases, suggesting a role for angiogenesis. The aim of this study is to investigate whether the presence of a primary CRC is associated with changes in angiogenic status and proliferation/apoptotic rate in synchronous liver metastases and/or adjacent liver parenchyma.

Methods: Gene expression and localization of CD31, HIF-1α, members of the vascular endothelial growth factor (VEGF) and Angiopoietin (Ang) system were studied using qRT-PCR and immunohistochemistry in colorectal liver metastases and nontumorous-adjacent liver parenchyma. Proliferation and apoptotic rate were quantified. Three groups of patients were included: (1) simultaneous resection of synchronous liver metastases and primary tumor (SS-group), (2) resection of synchronous liver metastases 3 to 12 months after resection of the primary tumor [late synchronous (LS-group)], and (3) resection of metachronous metastases >14 months after resection of the primary tumor (M-group).

Results: In all 3 groups a higher expression of the angiogenic factors was encountered in adjacent liver parenchyma as compared to the metastases. VEGFR-2 gene expression was abundant in adjacent liver parenchyma in all 3 groups. VEGF-A and VEGFR-1 were prominent in adjacent parenchyma in the SS-group. The SS-group showed the highest Ang-2/Ang-1 ratio both in the metastases and the adjacent liver. This was accompanied by a high turnover of tumor cells.

Conclusion: In the presence of the primary tumor, the liver parenchyma adjacent to the synchronous liver metastases provides an angiogenic prosperous environment for metastatic tumor growth.

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Metastatic disease is the major cause of death in cancer patients. The detection of metastases can vary from many years after resection of the primary tumor to the presence of clinically detectable metastases before a primary tumor can be identified, despite extensive clinical and radiological investigations. This well-recognized clinical entity is called "carcinoma of unknown primary" and is associated with a life expectancy of only 2 to 3 months.[1,2] Patients presenting with a primary tumor and simultaneous metastases—so called synchronous metastases—form an intermediate category with respect to the above mentioned time span between primary tumor and detection of metastases.

Colorectal carcinoma (CRC) is one of the leading causes of cancer-related death worldwide.[3] At the time of diagnosis, 20% of the patients present with synchronous liver metastases, and 30% of the patients will develop clinically detectable metastases afterwards, metachronous metastases. Partial liver resection is the only intentionally curative treatment of patients with colorectal liver metastases with a 5-year overall survival from 20% to 40%.[4] Patients who develop liver metastases after a long time interval after resection of the primary tumor have a better prognosis after partial liver resection than patients with synchronous liver metastases.[5,6]

Already in 1889 Paget postulated his seed and soil theory in which the interaction of the seed (the cancer cell) and the soil (the secondary organ) dictates the progression from micrometastases (also called clinically occult metastases or dormant metastases) to clinically detectable macrometastases.[7–9] The development of new blood vessels—angiogenesis—is considered to be one of the essential prerequisites for the progression from nonangiogenic dormant micrometastases to a macrometastasis with an angiogenic phenotype.[10] Accumulating evidence suggests that a primary tumor can influence the distant metastatic site to create a more prosperous environment to enhance the take of a metastatic tumor deposit in the distant organ.[11] In mouse tumor models it was demonstrated that bone marrow derived haematopoietic cells, expressing vascular endothelial growth factor receptor-1 (VEGFR-1), colonize pre-metastatic niches to prepare the soil before metastatic tumour cells arrive.[12] Subsequently, VEGFR-2 expressing circulating endothelial progenitor cells and tumor cells settle in these premetastatic niches. Also in humans with cancer, VEGFR-1 positive clusters were shown to be present in lymph nodes and lung, not only concomitant with metastatic deposits in these tissues but also in tissues not (yet) invaded by metastases.[12] These data suggest that the primary tumor is able to initiate a prosperous environment in distant tissues to favour metastatic tumor progression. The opposite effect—inhibition of the distant metastases by the primary tumor—is also well recognized. In mouse models, pulmonary metastases demonstrated a rapid progression after removal of the primary tumor, which was considered to be the result of elimination of the antiangiogenic compound angiostatin produced by the primary tumor.[13–15] After resection of the primary tumor the antiangiogenic effects disappear, and the metastases undergo an "angiogenic switch" leading to angiogenesis and enhanced tumor growth.[14] Also in humans, positron emission tomography and biopsies of liver metastases during primary tumor resection, suggest that primary tumor resection can induce progression of metastases.[15–18]

In this study, we test the hypothesis that colorectal liver metastases and/or adjacent nontumorous liver parenchyma are influenced by the primary tumor with respect to angiogenic make-up and proliferation status. To this end we analyzed patients with synchronous liver metastases removed simultaneously with the primary CRC (the

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SS group), patients with synchronous liver metastases removed 3 to 12 months after resection of the primary CRC (the late synchronous (LS) group), and patients with metachronous liver metastases (the M group) in whom liver metastases were detected and resected later than 14 months after resection of the primary tumor. We quantified gene expression levels of the VEGF-A family and the Angiopoietin (Ang)-Tie-2 system: VEGF-A, VEGFR-1, VEGFR-2, Placental growth factor (PlGF), Ang-1, Ang-2, and their tyrosine kinase receptor Tie-2, and the hypoxia controlled transcription factor HIF-1α in liver metastases and adjacent parenchyma using quantitative RT-PCR. The cellular localization of the proteins was analyzed by immunohistochemistry.

MATERIALS AND METHODS

Patients and Tissue Samples

All procedures and use of anonymized tissues were performed according to national guidelines. Tissue samples of 29 patients who had undergone liver surgery because of colorectal liver metastases were collected. Samples were taken at the periphery of the metastatic nodule to avoid necrotic tumor areas. The lesions were classified as synchronous liver metastases resected simultaneously with the primary tumor (SS group, n = 6, 4 solitary), synchronous liver metastases resected between 3 and 12 months after resection of the primary tumor (LS group, n = 10, 7 solitary) and metachronous liver metastases (M group, n = 13, 11 solitary) diagnosed and resected 14 months or more after the removal of the primary colorectal tumor. In patients with synchronous liver metastases, the metastases were detected before the resection of the primary tumor by ultrasound or computer tomography scanning. Patient and tumor characteristics including the clinical risk score according to Fong et al are described in Table 1.[6] Samples from histological normal livers (n = 9), obtained from surplus donor liver, or partial liver resection for benign disorders were used as a reference and not for statistical comparisons.

The same tissue blocks were used both for immunohistochemistry and for real-time RT-PCR to quantify gene expression. For real-time RT-PCR analysis, the metastatic tissue was separated from the adjacent nontumorous liver parenchyma to distinguish the gene expression pattern in both tissue types.

TABLE 1. Patient and Tumor Characteristics

Clinicopathological Characteristic	No of Patients: 29
Sex	
Female	12 (41%)
Male	17 (59%)
Age	
Average	65 years
Range	44–77 years
Primary tumor site	
Right colon	8
Left colon	16
Rectum	5
Synchronous/metachronous	
Simultaneous synchronous	6
Late synchronous	10
Metachronous	13
Median diameter (IQR) of largest metastasis (cm)	
Simultaneous Synchronous	5.4 (9.4)
Late synchronous	4.5 (7.3)
Metachronous	7.5 (3.0)
Single/multiple metastases	
Single	22 (76%)
Multiple	7 (24%)
Clinical risk score*	
Average	2.2
SD	1.0

*This score is based on the following variables which—if present—contribute to a worse survival: positive resection margin, extra hepatic disease, node-positive primary tumor, disease-free interval from primary tumor resection to metastases detection, number of liver metastases >1, size of largest metastases >5 cm, and carcinoembryonic antigen serum level (>200 ng/mL).

Gene Expression Analysis by Real-Time Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated as described earlier.[19] Exons overlapping primers and minor groove binder probes used for real-time reverse transcription-polymerase chain reaction (RT-PCR) were purchased as Assay-on-Demand from Applied Biosystems (Nieuwekerk a/d IJssel, The Netherlands, Table 2). All PCR reactions were performed in duplicate. Distilled water was analyzed to exclude unspecific signals arising from impurities and consistently showed no amplification signals. *Taq*Man PCR was performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems) as previously described.[19,20] Gene expression was normalized to the expression of the housekeeping gene GAPDH.

Immunohistochemistry to Identify Cellular Location of Protein Expression

All antibodies and concentrations used for immunohistochemistry are summarized in Table 2.

For VEGF-A, VEGFR-1, VEGFR-2, Ang-1, Ang-2, and Tie-2, 5 μm sections of frozen tissues were used as described previously.[20] CD31, CD34, αSMA, Caspase-3a, and Ki-67 were stained on formalin fixed tissue of 5 μm. Single immunohistochemical staining with anti-CD31 was performed to detect the vascular pattern in the metastases and their adjacent liver parenchyma, as described earlier.[21] Double staining of CD34 with αSMA and Ki-67 with Caspase-3a were performed to determine the presence of pericyte coverage of microvessels and proliferation rate versus apoptotic rate, respectively.[22]

Evaluation of Immunohistochemical Staining

The number of proliferating and apoptotic cells was quantified on Ki-67/Caspase-3a double stained sections using an $\times$ 400 microscopic field. In nontumorous adjacent liver parenchyma at least 500 hepatocytes were counted. In the most vital outer zone of the metastases at least 1,000 tumor cells were counted. The results are presented separately as the percentage of positively stained cells for adjacent liver parenchyma and the metastases. Using the double stained slides we quantified the number of apoptotic and proliferating cells in the same tissue region, preventing the potential bias of sampling error. Especially in the metastases, tumor heterogeneity could induce a bias if proliferation and apoptosis are scored in different tumor sections. As a surrogate marker of tumor growth we calculated the proliferation/apoptosis ratio.

STATISTICS

Quantitative data were expressed as mean $\pm$ SD or median with interquartile range (IQR). Comparisons between the groups were analyzed by the ANOVA with the Bonferroni post hoc test and Wilcoxon test. A P value of less than 0.05 was considered statistically significant.

RESULTS

CD31 Expression in Adjacent Liver Tissue is Higher Than in Metastases in Patients with Synchronous Liver Metastases

Gene expression levels of CD31 and CD34 were comparable in the liver metastases and therefore CD34 data are not shown. CD31 gene expression level in nontumorous adjacent liver parenchyma in both synchronous groups was higher as compared to the corresponding liver metastases (Fig. 1A). In the SS and LS group a 4-fold and 2-fold higher expression was found respectively ($P < 0.05$, Fig. 1A). The 3 types of metastases showed comparable CD31 expression levels (Fig. 1A).

Whereas sinusoidal CD31 protein expression in liver parenchyma is normally only seen in the periportal region, liver parenchyma immediately adjacent to the metastases uniformly showed upregulation of sinusoidal CD31 expression besides its presence in the periportal region (Fig. 1B).

Adjacent Liver Parenchyma Exhibits the Most Prosperous Angiogenic Environment in Simultaneously Resected Synchronous Liver Metastases

Analysis of the VEGF/VEGFR system revealed no differences in gene expression level of VEGF-A, VEGFR-1, VEGFR-2, and HIF-1α between the metastases in the SS, LS and M group (Fig. 2).

TABLE 2. Primers and Antibodies Used for qRT-PCR and Immunohistochemistry

	Primers		Immunohistochemistry	
	Assay ID	**Antibody**	**Dilution**	**Company**
CD31	Hs00169777_m1	Mouse anti–CD31	1/25*	Dako
VEGF–A	Hs00173626_m1	Rabbit anti–VEGF-A	1/100*	Santa Cruz
VEGFR–1	Hs01052936_m1	Rabbit anti–VEGFR–1	1/100*	Abcam
VEGFR–2	Hs00176676_m1	Rabbit anti–VEGFR–2	1/100*	Abcam
Ang–1	Hs00181613_m1	Goat anti–Ang–1	1/100*	Santa Cruz
Ang–2	Hs00169867_m1	Goat anti–Ang–2	1/50*	Santa Cruz
Tie–2	Hs00176096_m1	Rabbit anti–Tie–2	1/50*	Santa Cruz
HIF–1α	Hs00936366_m1			
PlGF	Hs00182176_m1			
GAPDH	Hs99999905_m1			
CD34		Mouse anti–CD34	Ready to use	Immunotech
Ki–67		Mouse anti–Ki67	1/100	Dako
Caspase 3a		Rabbit anti–Caspase 3a	1/50	Cell signaling
αSMA		Mouse anti–αSMA	1/80	Immunotech
		HRP–conjugated rabbit anti–mouse Ig	1/100†	Dako
		HRP–conjugated anti–rabbit Ig	1/100†	Dako
		HRP–conjugated anti–goat Ig	1/100†	Dako

HRP indicates horseradish peroxidise; Ig, immunoglobulins.
*Diluted in 1% BSA/PBS.
†Diluted in 1% BSA/PBS + 1% human serum.

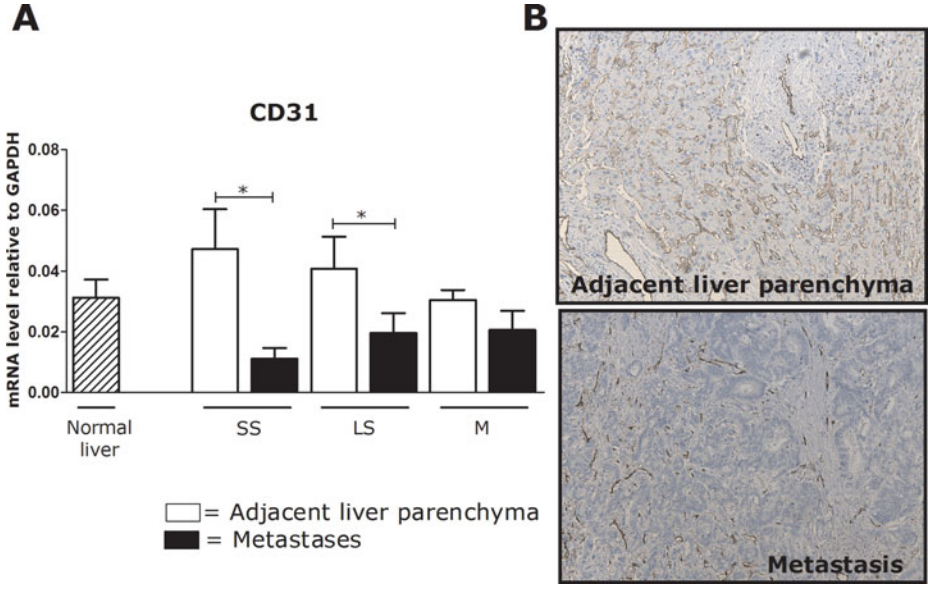

FIGURE 1. Higher CD31 gene expression in adjacent liver parenchyma in the 2 synchronous groups. Relative gene expression of CD31 in colorectal liver metastases and their adjacent liver parenchyma, with normal liver as a reference. A, Endothelial gene CD31 expression is significantly higher in the nontumorous adjacent liver parenchyma of the synchronous and late synchronous groups. The level of expression of CD31 was similar in all 3 metastases groups. Simultaneous synchronous (SS) n = 6, late synchronous (LS) n = 10, metachronous (M) n = 13. B, Immunohistochemical staining with CD31 of colorectal liver metastases and the adjacent liver parenchyma. The tumor is poorly vascularised compared to the nontumorous adjacent liver parenchyma. (Original magnification 200 ×) *$P < 0.05$

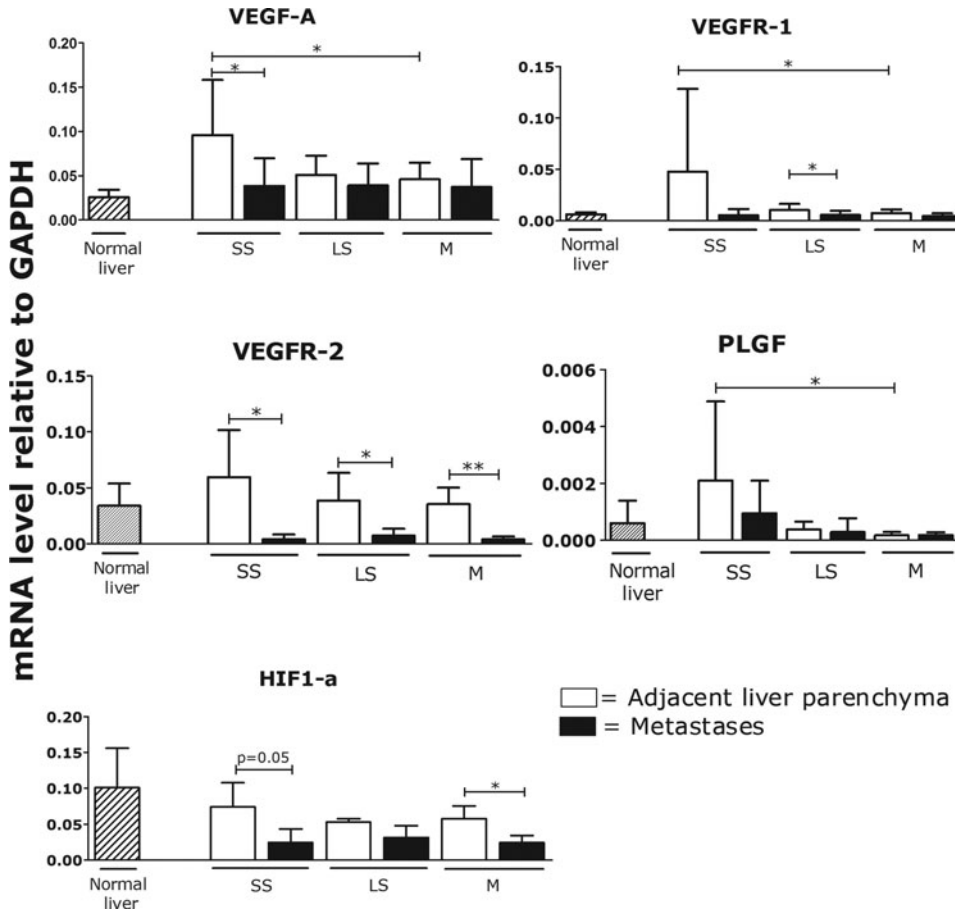

FIGURE 2. The adjacent liver parenchyma of the SS group has a higher gene expression of VEGF-A, VEGFR-2, and HIF1-α compared to the metastases. HIF-1α and VEGF-A and its receptors mRNA expression levels were determined by real-time RT-PCR as described in materials and methods. HIF-1α was less abundantly expressed in metastases compared to adjacent liver parenchyma in all 3 metastases groups, showing a borderline significantly lower and a significantly lower expression in the simultaneous synchronous (SS) and metachronous (M) metastases. VEGFR-2 was higher expressed in the adjacent liver parenchyma's of all metastases, whereas VEGF-A was only significantly higher expressed in adjacent liver parenchyma of the SS group metastases. The adjacent liver parenchyma of this group also showed a higher expression of VEGF-A compared to the adjacent liver parenchyma of the metachronous metastases. Simultaneous synchronous (SS) n = 6, late synchronous (LS) n = 10, metachronous (M) n = 13. Normal liver is shown as a reference. *$P < 0.05$, **$P < 0.001$

In contrast, VEGF-A and VEGFR-1 gene expression in adjacent liver parenchyma in the SS group was approximately 2.5- and 10-fold higher, respectively, than in metachronous adjacent liver parenchyma (Fig. 2). Also the gene expression of PlGF in adjacent liver parenchyma in the SS-group was approximately 10-fold higher than in metachronous adjacent liver parenchyma ($P < 0.05$; Fig. 2). In the LS and M group VEGF-A expression was comparable in parenchyma and the corresponding metastases. VEGFR-2 gene expression was always higher in adjacent liver parenchyma as compared to the corresponding metastases with the highest expression in the adjacent liver parenchyma of the SS metastases. The latter demonstrated a 14-fold higher expression in adjacent liver parenchyma as compared to the metastases. A 3-fold higher HIF-1α expression was seen in the adjacent liver parenchyma compared to the metastases in the SS group ($P = 0.05$).

Using immunohistochemistry, VEGF-A and VEGFR-1 protein were detected in stromal cells and tumor cells in all 3 types of metastases. VEGFR-2 was expressed in tumor cells and endothelial cells in the tumor vessels. VEGF-A, VEGFR-1, and VEGFR-2 showed a sinusoidal expression pattern in the liver parenchyma. VEGF-A and VEGFR-2 expression was more pronounced in adjacent liver parenchyma than in the metastases (Fig. 5).

Adjacent Liver Parenchyma and Metastases of the Simultaneous Synchronous Group Showed a Balance Toward Angiogenesis

Analysis of the factors of the Angiopoietin system did not reveal any differences in gene expression levels of Ang-1, Ang-2, and Tie-2 between the 3 types of metastases (Fig. 3). The Ang-2/Ang-1 ratio, representing the net angiogenic effect of the Angiopoietins, was however 3-fold higher in the SS metastases compared to the LS metastases and 4.5-fold higher than in the metachronous metastases. In the adjacent liver parenchyma, the Ang-2/Ang-1 ratio was 2-fold lower as compared to its metastases in the SS group. The gene expression level of Ang-2 itself in the adjacent liver parenchyma was about 3-fold higher than in the metastases in the SS group. In the LS and M group, Ang-2 expression was comparable in the adjacent liver parenchyma and metastases (Fig. 3).

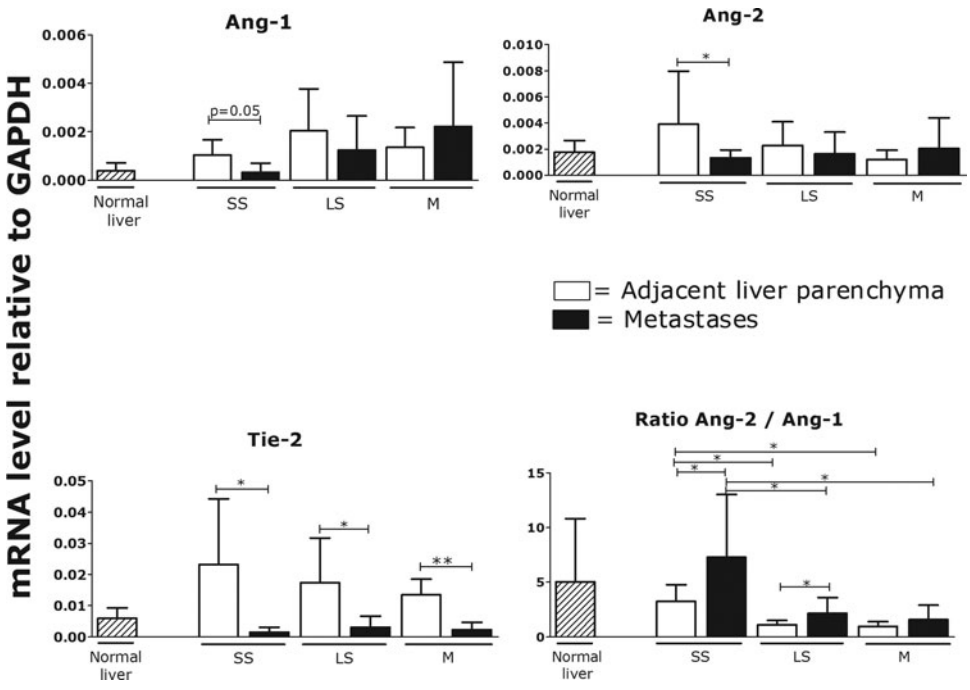

FIGURE 3. Higher expression of Ang-2 and its Tie-2 receptor in adjacent liver parenchyma of the SS group. Angiopoietins and their tyrosine kinase receptor Tie-2 mRNA expression levels were determined by real-time RT-PCR as described in materials and methods. A significantly higher expression of Ang-2 and its Tie-2 receptor was seen in adjacent liver parenchyma of the simultaneous synchronous (SS) group. Looking at the Ang-2/Ang-1 ratio in the metastatic compartment of the SS metastases this was most pronounced compared to the other metastases. Also the adjacent liver parenchyma of the SS group had a higher Ang-2/Ang-1 ratio than the adjacent liver parenchyma of the other 2 groups. Simultaneous synchronous (SS) n = 6, late synchronous (LS) n = 10, metachronous (M) n = 13. Normal liver is shown as a reference. *$P < 0.05$, **$P < 0.001$

Tie-2 expression was higher in the adjacent liver parenchyma compared to the corresponding metastases in all 3 groups. Remarkably, the largest difference in expression levels between adjacent parenchyma and metastases was seen in the SS group with a 16-fold higher expression in adjacent liver parenchyma, whereas in the LS and M group this was 5.5- and 6-fold, respectively. The Ang-2/Ang-1 ratio in adjacent liver parenchyma of the SS group was 3-fold higher than in the adjacent parenchyma of the LS and M group. Immunohistochemical staining of Ang-2 and Tie-2 showed a sinusoidal expression pattern in adjacent liver parenchyma. This staining was more pronounced in the parenchyma as compared to the metastases in which not only endothelial cells were positively stained but also tumor and stromal cells (Fig. 5). Ang-2 and Tie-2 expression were comparable in the SS, LS, and M groups, both in the metastases and the parenchyma.

Mature Blood Vessels in the Colorectal Liver Metastases

A marker of vessel maturation is the coverage of blood vessels by pericytes.[23] We semiquantitatively analyzed the maturation of the vessels in the 3 groups of patients by performing a double staining for the endothelial cells of vessels and the pericytes using CD34 and αSMA. The predominant pattern in all 3 types of metastases is that of vessels strongly covered by αSMA positive pericytes, suggesting mature vessels being the major phenotype in the metastases (Fig. 6). In adjacent liver parenchyma strong αSMA coverage was observed in nearly all sinusoids. This is compatible with increased expression of αSMA as can be found both in acute and chronic liver diseases.[24]

Higher Apoptotic Rate and Lower Tumor Growth Rate in Simultaneously Resected Synchronous Liver Metastases

Tumor cell turnover was highest in the SS group as reflected by a higher rate of both apoptotic and proliferating tumor cells. Rate of apoptosis was about 1.5-fold higher in the SS group as compared to the LS and M group ($P = 0.05$; Fig. 4B) with a 20% higher proliferation rate in the SS group as compared to the LS group ($P = 0.05$; Fig. 4B). As a surrogate marker of tumor growth we calculated the ratio Ki67 positive cells/caspase 3a positive cells. It seemed that the tumors in the SS group had the lowest growth rate (Fig. 4C).

DISCUSSION

The main finding of this study is that in patients in whom the primary tumor is still present, liver parenchyma adjacent to the liver metastases provides a highly prosperous angiogenic environment. In contrast, we encountered no differences in VEGF/Ang/Tie 2 levels in the liver metastases itself. An increased expression of VEGF-A, PlGF and their receptor VEGFR-1 was seen in adjacent liver parenchyma of patients in the SS group as compared to patients in the M group. When using the Ang-2/Ang-1 ratio we encountered a shift favoring angiogenesis both in adjacent liver parenchyma and the metastases in the SS group as compared to the LS and M groups. This was accompanied by high Tie-2 levels in adjacent parenchyma as compared to liver metastases.

Because the vascular pattern of adjacent liver parenchyma and metastases can differ, we used both CD31 and CD34 staining to determine the vascularization of both tissues. In contrast to tumor

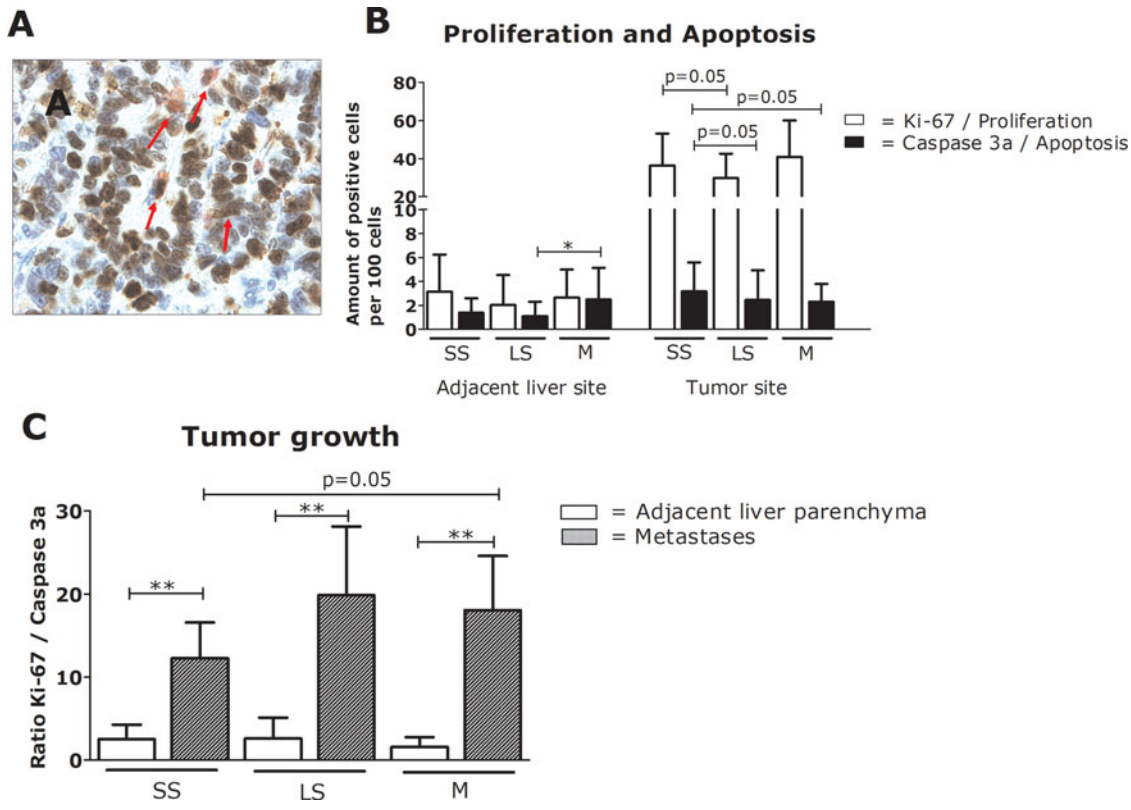

FIGURE 4. No rapid outgrowth of liver metastases after resection of the primary colorectal tumor. The balance and the rate of proliferation and apoptosis were determined by counting the positive cells via double staining of Ki-67 and Caspase-3a (A; red arrows are caspase-3a positive cells) as described in the materials and methods. B, In the balance of proliferation and apoptosis there is a borderline significant difference between the Synchronous groups, with a higher amount of proliferation and apoptosis in the simultaneous synchronous compared to the late synchronous metastases. In the adjacent liver there was a higher amount of apoptosis in the metachronous group compared to the late synchronous metastases. C, Calculating the Ki-67/Caspase-3a rate to determine tumor growth showed that the metachronous metastases had a borderline significantly higher tumor growth rate compared to the simultaneous synchronous metastases. Simultaneous synchronous (SS) group n = 6, late synchronous (LS) group n = 10, and metachronous (M) group n = 13. $^{**}P < 0.001$

blood vessels, liver sinusoids hardly express CD34 and CD31.[25,26] However, sinusoids in liver parenchyma adjacent to tumor exhibits upregulation of CD31 and CD34.[20,19] Because no major differences in CD31 and CD34 expression were detected neither in the liver metastases nor in the liver parenchyma we only presented CD31 data. As expected we found that the metastases were poorly vascularised compared to the adjacent liver parenchyma.

We investigated 3 groups of patients with different time intervals of resection of the liver metastases after resection of the primary tumor. One group of patients underwent a simultaneous resection of the primary tumor and the synchronous liver metastases representing liver metastases, which had grown under the influence of the primary tumor. The second group consisted of patients who also had synchronous liver metastases but in whom the primary tumor was resected several months before the resection of the liver metastases representing liver metastases growing independently of the primary tumor. The third group consisted of patients in whom liver metastases were detected at least 14 months after the resection of the primary tumor. The exact time frame of metastatic tumor development in relation to primary tumor resection is not feasible, because that would require repeated biopsies of the metastases and adjacent liver parenchyma at various time points.

The development of colorectal liver metastases is a complex process.[8] After detachment from the primary tumor, tumor cells arrive in the liver and establish crosstalk with liver parenchyma thereby creating their own microenvironment.[7] Metastatic cell proliferation and growth will only ensue if the micrometastases and a permissive environment undergo a switch to the angiogenic phenotype.[10] The primary tumor can have an active role in making the liver permissive for future metastases by recruitment of VEGFR-1 expressing haematopoietic progenitor cells, which initiate the premetastatic niche.[12,27] These authors also demonstrated that conditioned media obtained from tumors contributed to the metastatic potential, which is probably mediated via VEGF and placental growth factor (PlGF).[12] An active role for VEGFR-2 in making the liver permissive for the homing and growth of metastases was corroborated in a study in which administration of a VEGFR-2 inhibitor inhibited the development of liver metastases in a mouse model.[28] Also our data showed a high expression of VEGF-A, VEGFR-1, VEGFR-2, and PlGF in adjacent liver parenchyma of the SS group and therefore it is conceivable that liver parenchyma in this group is indeed permissive. PlGF contributes to angiogenesis because it shares its receptor (VEGFR-1) with VEGF-A and thus can act as a competitor to VEGF-A/VEGF-R1 binding, leaving VEGF-A for ligation to and activation of VEGFR-2.[29] Furthermore, it has been

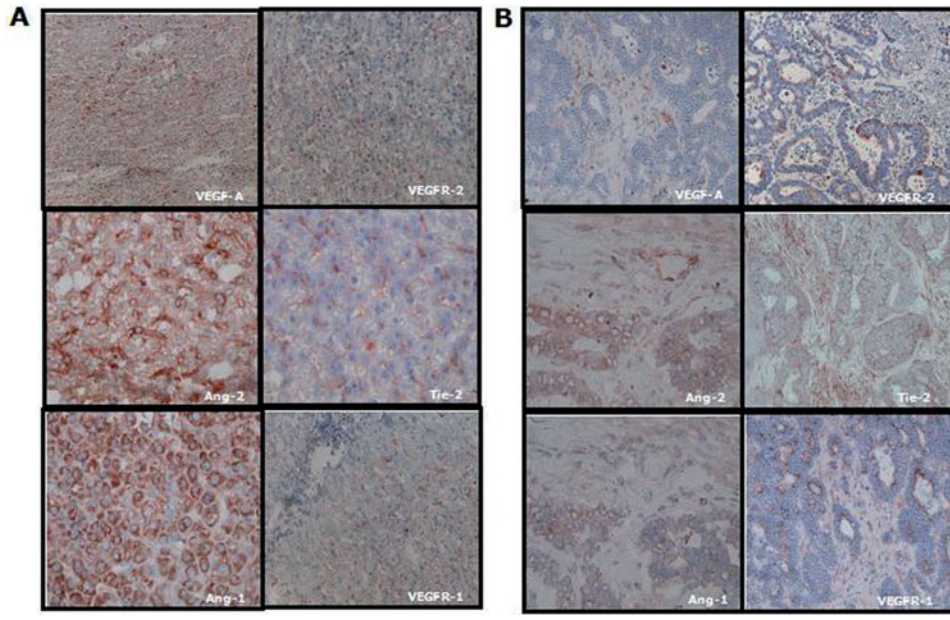

FIGURE 5. Localization of VEGF-A, its receptor VEGFR-2, Ang-2 and its receptor Tie-2 expression in the adjacent liver parenchyma (A) and metastases tissue (B) of colorectal liver metastases. A, VEGF-A had a more pronounced positive staining in the adjacent liver parenchyma than in the metastases (B). VEGFR-2 staining in the adjacent liver parenchyma was seen in a sinusoidal endothelial expression pattern whereas in the metastases positive staining of this receptor was seen in tumor cells, blood vessels and stromal cells. Ang-2 and its receptor Tie-2 were also seen in a sinusoidal pattern in the adjacent liver parenchyma with also positive blood vessels in the metastases as well as positive tumor and stromal cells.

described that PlGF can recruit VEGFR-1$^+$ myeloid bone marrow cells which indirectly promote angiogenesis by secreting proangiogenic cytokines.[30] On top of that, animal and cell culture studies showed that the VEGF/PlGF heterodimer can activate VEGFR-1 to become proangiogenic.[31] Applying these findings to our study might suggest that adjacent liver parenchyma stimulates the angiogenic signaling via the VEGFR-2 and the VEGFR-1 signaling pathway.[32,31]

On the basis of the fact that VEGFR-1 and Tie-2 are also predominantly found in bone marrow-derived cells it might well be that adjacent liver parenchyma is probably providing a prosperous environment via both angiogenesis as well as vasculogenesis. Further studies using markers specifically for bone marrow-derived cells are necessary to unravel these mechanisms.

The second major finding of our study is that in the SS group the highest Ang-2/Ang-1 ratio both in the metastases and adjacent parenchyma was observed. This high Ang-2 gene expression in adjacent liver parenchyma compared to the metastases in combination with the high VEGF-A, VEGFR-1, VEGFR-2 and PlGF gene expression provides also support to the concept of a prosperous niche. Contributing to that is the fact that Ang-1 and Ang-2 and their receptor Tie-2 have an essential role in vascular development and angiogenesis in which Ang-1 binding leads to vascular stabilization and Ang-2 binding to vessel destabilization, endothelial sprouting, and angiogenesis.[33–35] A high Ang-2/Ang-1 ratio as a reflection of angiogenesis and vascular remodeling is nowadays accepted and often used throughout the body in different pathological circumstances including brain, breast and liver cancer.[36–41]

The third main finding of our study is a high turnover of tumor cells in the SS group as is reflected by a high proliferation rate as well as a high apoptotic rate in tumor cells resulting in a low tumor growth rate. This is compatible with studies in mice, which also suggest a high turnover of tumor cells in the metastases.[13,42] In humans the apoptotic rate in colorectal liver metastasis is variable and ranges from 63%[43] to 3%.[44,45] The latter number is in agreement with our findings.

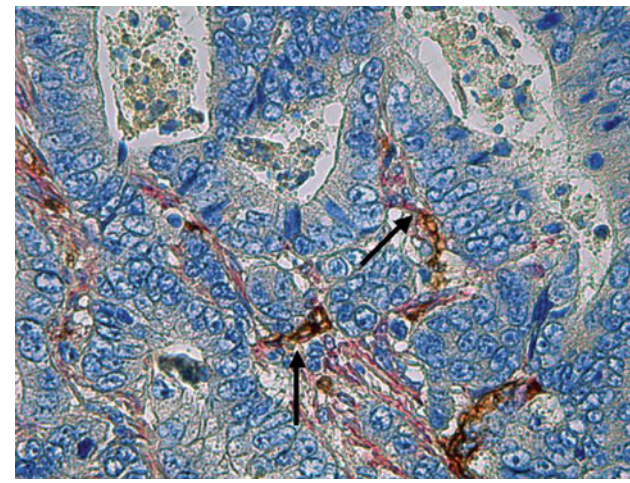

FIGURE 6. Double staining of αSMA (Fastred-staining) and CD34 (DAB-staining) to investigate the maturation status of the blood vessels. Example of a double stained blood vessel is presented by the black arrows. Vessels covered with αSMA positive pericytes are the most dominant feature in the metastatic tissue of all 3 types of metastases.

CRCs are believed to be angiogenic in a VEGF/VEGFR–Ang/Tie-2 dependent manner.[46,47] In this study, we demonstrate that adjacent liver parenchyma is a prosperous environment for the

development of liver metastases and we provide evidence that angiogenic molecules contribute to this. There could also be a role for the vascular cell adhesion molecules such as the endothelium (of endothelial cell) specific E-selectin. Recent studies showed that selectin-mediated interactions may contribute to the formation of a permissive microenvironment for metastasis.[48] The presence of E-selectin ligands on colorectal cancer cells correlates with enhanced adhesion to activated endothelial cells.[49,50] The binding of E-selectin to the tumor cells alters the gene expression profile of cancer cells to a survival and proliferation state through activation of p38 and ERK MAP kinases in which the latter is involved in the VEGFR-2 growth factor signaling.[51,52] Selectin-mediated signaling is also followed by upregulation of integrins.[53,54] Integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$ can induce activation of the angiopoietin–Tie-2 signaling and thereby induce angiogenesis.[55] It is known that E-selectin is expressed and upregulated on activated liver sinusoidal endothelial cells in response to metastatic tumor cells[56,57] and therefore might play a role in making the adjacent liver parenchyma permissive for metastatic growth via the above described pathways. The role of selectins in the formation of a permissive niche, however, necessitates further investigation.

Concluding, in patients with colorectal liver metastases in whom the primary tumor is still present, adjacent liver parenchyma—the soil—behaves as an angiogenesis driven prosperous environment for metastatic growth. Possibly the application of drugs targeting not only the VEGF-A/VEGFR-1 system but also the Ang-Tie system in both the tumor as well as in the metastatic niche might become a strategy to prevent metastatic tumor growth.[58,59]

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