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Progressive loss of endothelial P-selectin expression with increasing malignancy in colorectal cancer

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Adhesion of inflammatory cells to vascular endothelium is mediated by specific cell adhesion receptors on both leukocytes and endothelial cells. One of the adhesion molecules on the endothelium is P-selectin. Decreased vascular P-selectin expression has been associated with tumor progression in melanoma patients. We now report on the expression of endothelial P-selectin in colorectal cancer (CRC). We studied a colorectal tissue specimen series ranging from normal colorectal tissue via unmetastasized primary tumors to tumors with the same depth of invasion at the primary site but with liver metastases. Moreover, P-selectin expression levels in liver metastases were determined. The number of P-selectin positive vessels as a fraction of the total number of vessels, both intra- and peritumorally, was determined by staining for CD62P and CD34, respectively. Furthermore, by immunostaining for leukocytes (CD45) and macrophages (CD68), it was evaluated whether levels of P-selectin expression influenced infiltrate density and composition. The results showed that levels of peritumoral P-selectin expression were reciprocal to the degree of progression in CRC. This relation was even more pronounced intratumorally: in metastasized primary tumors and in the metastatic lesions, P-selectin expression was virtually absent. This distribution pattern was reflected in the numbers of leukocytes that accumulated in the various tissues, since in the primary tumors with metastases, and in the metastatic lesions, hardly any infiltrating cells were observed. In these lesions, leukocytes were present in the peritumoral zone, but seemed unable to enter the tumor tissue. In primary tumors without metastasis, the intratumoral leukocyte infiltration density was significantly higher. Recruitment levels of macrophages remained constant throughout the different tissues. We suggest that downregulation of endothelial P-selectin expression is a mechanism by which CRC lesions evade inflammatory regression and, thereby, progress to a more advanced stage of malignancy.
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Colorectal cancer (CRC) is one of the most common malignancies in the Western world.[1] Factors associated with the outcome of CRC, such as angiogenesis and leukocyte infiltration, have been studied extensively.[2–5] Leukocyte infiltration is initiated by extravasation of leukocytes as the result of an appropriate expression of cell surface adhesion receptors both on leukocytes and endothelial cells. Firm adherence of leukocytes to microvascular endothelial cells finally results in diapedesis of leukocytes across the endothelial lining and penetration, in the case of a tumor, into the surrounding malignant tissue.[6–9]

Among endothelial cell adhesion molecules, the selectin family plays a crucial role in the adhesion of leukocytes to activated endothelium.[7,10,11] An important member of this family is P-selectin. P-selectin promotes immediate weak interaction and rolling of leukocytes along the vascular endothelium.[12] In the human melanoma progression series including benign, malignant and metastatic lesions, we demonstrated that the degree of vascular P-selectin expression is inversely related to the level of progression.[13] There are indications that in primary colorectal tumors endothelial P-selectin expression may be downregulated as well.[14] Various other studies have reported expression of adhesion molecules on endothelial cells within other primary human tumors.[9,15,16] Only a few studies addressed

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the level of expression of these molecules in metastatic lesions.[13,17] Endothelial P-selectin expression in metastases of colorectal carcinoma has not been investigated at all. In the present study, we investigated P-selectin expression in a series of human colorectal tissue specimens encompassing normal colorectal tissue, primary tumors without metastates to lymph nodes or liver, and colorectal tumor specimens with the same invasion depth at the primary site but with liver metastases. Moreover, P-selectin expression levels in liver metastases were determined. Furthermore, we investigated the relation between the expression of P-selectin and the presence of inflammatory cells, both bearing the P-selectin ligand and expressing other adhesion molecules. It has been demonstrated that in CRC, inflammatory cells are predominantly distributed along the invasive margin of the tumor.[6,18] Accordingly, the relation between P-selectin expression and the number of infiltrating cells was investigated in two different areas of the tumor: both in the invasive margin and within the tumor lesion itself. Our findings indicate that one of the mechanisms by which advanced colorectal lesions evade inflammatory regression is via a decrease of endothelial P-selectin expression.

Table 1 Characteristics of the patients

Number of patients	*CRC without metastases*	*CRC with metastases*
Total number	11	11
Males	7	4
Females	4	7
Age at surgery (year)		
Range	47–75	44–83
Mean	62	60
PTNM		
Tumor size		
T1	0	0
T2	0	0
T3	9	11
T4	2	0
Nodal status		
N0	11	2
N1–2	0	9
Metastatic disease		
M0	11	0
M1	0	11
Grade		
Well/moderate	11	11
Poor	0	0

Materials and methods

Patients

After informed consent, 22 patients were included in this study, and divided in the following groups: one group of 11 patients with CRC without metastases in lymph nodes or liver (stage T_{3-4}, N_0, M_0); a second group of 11 patients with a colorectal primary tumor with the same depth of invasion as the first group but with liver metastases (synchronous metastases, stage T_3, N_{0-2}, M_1); and a third group consisting of the metastatic tissue samples from these liver metastases. For comparison of results a fourth group of samples was analyzed consisting of normal colorectal tissue. Patient and tumor characteristics are given in Table 1. Representative tissue samples were taken from the CRC excision specimen and the corresponding liver metastases from patients who underwent surgery at the University Medical Centre Nijmegen, The Netherlands.

Patients did not receive any form of preoperative therapy (radiotherapy/chemotherapy) for the primary tumor and the liver metastases.

Based on conventional histopathological examination of paraffin sections, the pathological stage was determined according to the TNM classification.[19]

Immunohistochemistry

In all, 4 μm paraffin sections of normal colon, primary tumor and liver metastases were semiserially cut.

After air-drying overnight at 37°C, sections were deparaffinized in xylol and rehydrated in an alcohol gradient. Endogenous peroxidase activity was quenched by incubation with 0.01% H_2O_2 for 30 min followed by PBS washes. After endogenous avidin and biotin had been blocked in the liver metastases, sections were treated with pronase 0.1% at 37°C for 10 min and washed in PBS. After preincubation with PBS containing 20% normal goat serum, sections were incubated with polyclonal rabbit anti-human P-selectin, CD62P (JC/70A, dilution 1:400; Pharmingen, San Diego, CA, USA). Furthermore, sections were incubated with monoclonal antibody against human endothelial antigen CD34 (Qbend/10, dilution 1:750, Neomarkers, Fremont, CA, USA).

Various inflammatory cells were immunostained with the following antibodies: anti-CD45 (anti-human common leukocyte antigen, LCA, dilution 1:160), anti-CD68 (anti-human macrophage, Kpl, dilution 1:100) and anti-elastase (anti-human neutrophil elastase, NP57, dilution 1:800). The latter three antibodies were obtained from Dako A/S, Glostrup, Denmark).

Antibody binding was detected by subsequent incubation with, respectively, biotinylated goat anti-rabbit immunoglobulin (Ig) for P-selectin staining, and biotinylated horse anti-mouse Ig for all other stainings. The alkaline phosphatase complex was detected by fast red. Sections were counterstained with hematoxylin and mounted in Imsol.

Assessment of P-selectin Positive Vessels

Vascular density in normal colon, primary CRC with and without metastases and in liver metastases was studied in tissue sections that were stained with anti-CD34 monoclonal antibody.

Intratumoral and peritumoral vessel counts were determined in two randomly chosen, nonoverlapping $\times 100$ fields. Results were expressed as the average number of vessels per $\times 100$ field.

The proportion of vessels stained for P-selectin was determined as a fraction of the total number of CD34-positive vessels that was determined in serial tissue sections. Results were scored by two independent observers who were blinded to patients' characteristics, type of therapy and timing of biopsy. In cases where scores differed, slides were reviewed to reach consensus.

Analysis of Inflammatory Cells

In normal colon, primary CRC and liver metastases, both intratumorally and along the invasive margin, the numbers of leukocytes, macrophages and granulocytes were determined by staining with anti-CD45, anti-CD68 and anti-elastase antibody, respectively. Results were expressed as number of inflammatory cells per $\times 100$ field in serially cut tissue sections.

Statistical Analysis

Quantitative data were analyzed by independent *T*-testing. Correlation between the number of leukocytes and the relative P-selectin positivity was analyzed by using Pearson's product moment correlation coefficient. Significance was defined at $P = 0.05$.

All statistical analyses were performed with the SPSS statistical software package, version 9.0.0, for Windows (SPSS Benelux bv, Gorinchem, The Netherlands). Data are presented as mean plus and minus standard deviation (s.d.).

Results

Staining Characteristics

Vascular density in normal colon, CRC and synchronous liver metastasis was studied in tissue sections that were stained with anti-CD34 antibody.

In contrast to staining of normal colon and normal liver tissue, which showed widely separated vessels with intact vessel wall integrity, vascular staining of tumor tissue in both primary tumor and liver metastasis demonstrated elongated, strongly dilated and often irregularly shaped vessels, sometimes arranged in clusters (Figure 1). This pattern was identical both intratumorally and peritumorally.

Expression of P-selectin in tissue samples of normal colon, CRC and synchronous liver metastasis was determined by immunohistochemical analysis with anti-CD62P antibody. As shown in Figure 1, in normal colorectal tissue, P-selectin was strongly expressed on larger vessels, and delicate expression was observed on microcapillaries. In primary tumor lesions without metastases, the expression level was slightly decreased. In primary tumor lesions with metastases only a small part of the vessels was P-selectin positive, whereas in liver metastases, P-selectin was completely lacking.

Quantitative Analysis of Vascular Density and P-selectin Expression

Vascular density in both the intratumoral zone and the area along the invasive margin was assessed by counting the number of vessels per $\times 100$ field in tissue sections stained for CD34. In four patients, the diameter of the peritumoral zone of the liver metastases was very narrow or completely absent. Therefore, determining vascular density in a reliable quantitative fashion was not possible there. For this reason, these cases were excluded from analysis of the peritumoral zone.

Vascular densities, both intra- and peritumorally, are shown in Figure 2. In normal colorectal tissue, the mean number $\pm$ s.d. of vessels/field was 112 ± 67. In the intratumoral area of the colorectal primary tumor of patients without metastases to lymph nodes or liver, the mean number of vessels/$\times 100$ field was significantly lower: 60 ± 36 ($P = 0.035$). In contrast, in patients with liver metastases, the intratumoral area of the primary tumor had an increase in vascular density of 140 ± 37, which was significantly higher than patients without any metastases ($P = 0.001$). In the intratumoral area of the liver metastases, a lower vascular density was found: 79 ± 36. A similar pattern was found in the peritumoral areas, which contained 68 ± 39 vessels in colorectal primary tumors without metastases, 129 ± 32 in the colorectal tumor with synchronous liver metastases ($P = 0.001$) and 95 ± 37 in the peritumoral areas of the liver metastases.

The expression of endothelial P-selectin was determined as a fraction of the number of CD34-positive vessels in the colorectal specimen series, both intra- and peritumorally (Figure 3). In normal colon, $51 \pm 10\%$ of the vessels on the average were P-selectin positive whereas nonmetastazised primary tumors contained a significantly lower percentage of P-selectin-positive vessels in the intratumoral area ($31 \pm 19\%$, $P = 0.006$). In the peritumoral area of these lesions, the number of P-selectin positive vessels was 51 ± 14, which was significantly higher than the number of vessels in the intratumoral area ($P = 0.011$). Interestingly, in primary colorectal tumors of patients with liver metastases, the decrease of P-selectin expression was far more pronounced,

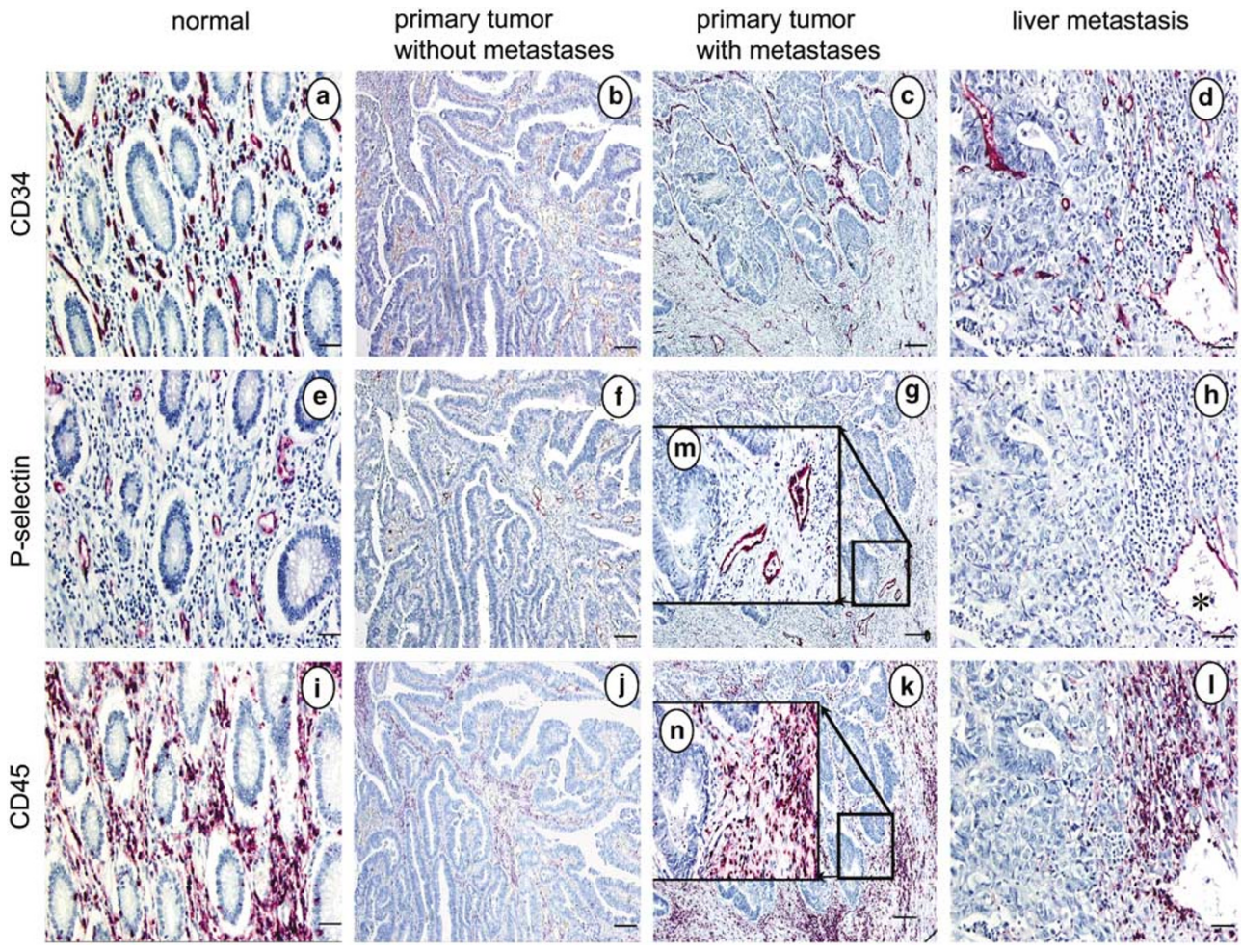

Figure 1 Immunohistochemical analysis of the expression of CD34 (**a–d**), P-selectin (**e–h**) and CD45 (leukocytes, **i–l**) in paraffin-embedded sections of serial sections of normal colon (**a**, **e**, **i**), primary CRC without metastases (**b**, **f**, **j**), primary CRC with metastases (**c**, **g**, **k**) and liver metastasis (**d**, **h**, **l**). Panels **m** and **n** show magnifications of parts of **f** and **j**, respectively. In **m**, P-selectin positive vessels are shown. Panel **n** shows leukocyte infiltration around these vessels. Note the absence of P-selectin-positive vessels and leukocytes intratumorally. The asterisk in **h** indicates a P-selectin-positive vessel in the peritumoral area of a liver metastasis, with an accumulation of leukocytes as demonstrated in **l**. Original magnification **b**, **f**, **j** (colorectal primary tumor without metastases) and **c**, **g**, **k** (colorectal cancer primary tumor with metastases) $\times 100$, with bar representing 100 μm. Original magnification **a**, **e**, **i** (normal colon) and **d**, **h** and **l** (colorectal liver metastasis) $\times 250$, with bar representing 40 μm.

both intra- and peritumorally: $6.6 \pm 9.6\%$ and $23.0 \pm 17.5\%$, respectively. Statistical analysis revealed that both in the intratumoral and the peritumoral area this decline was significantly higher than in patients without metastases ($P = 0.001$ and 0.001, respectively). A representative example of the relative absence of P-selectin staining of the tumor vessels within the primary colorectal lesion compared to the vessels in the surrounding tissue is shown in Figure 1.

A similar lack of expression could be demonstrated in liver metastases, with $2.8 \pm 5.0\%$ of the blood vessels positive for P-selectin in the intratumoral area of the liver metastases ($n = 11$) and $5.9 \pm 9.7\%$ positive vessels in the perilesional tissue ($n = 7$) (Figure 3).

Perilesional staining for P-selectin in the liver metastases was significantly lower compared to P-selectin staining of the primary tumor without and with metastases ($P = 0.0005$ and 0.033, respectively). Also, intralesional staining showed a significantly lower amount of P-selectin-positive vessels compared to the primary tumor tissue of patients without metastases ($P = 0.001$). In contrast, compared to primary tumor tissue of patients with synchronous liver metastases intralesional staining did not significantly differ as it was almost absent in the area in both groups.

Distribution of Inflammatory Cells

Next, the influx of different types of cells involved in the immune response was evaluated in relation to downregulation of endothelial P-selectin. The presence of leukocytes in normal colon tissue, primary

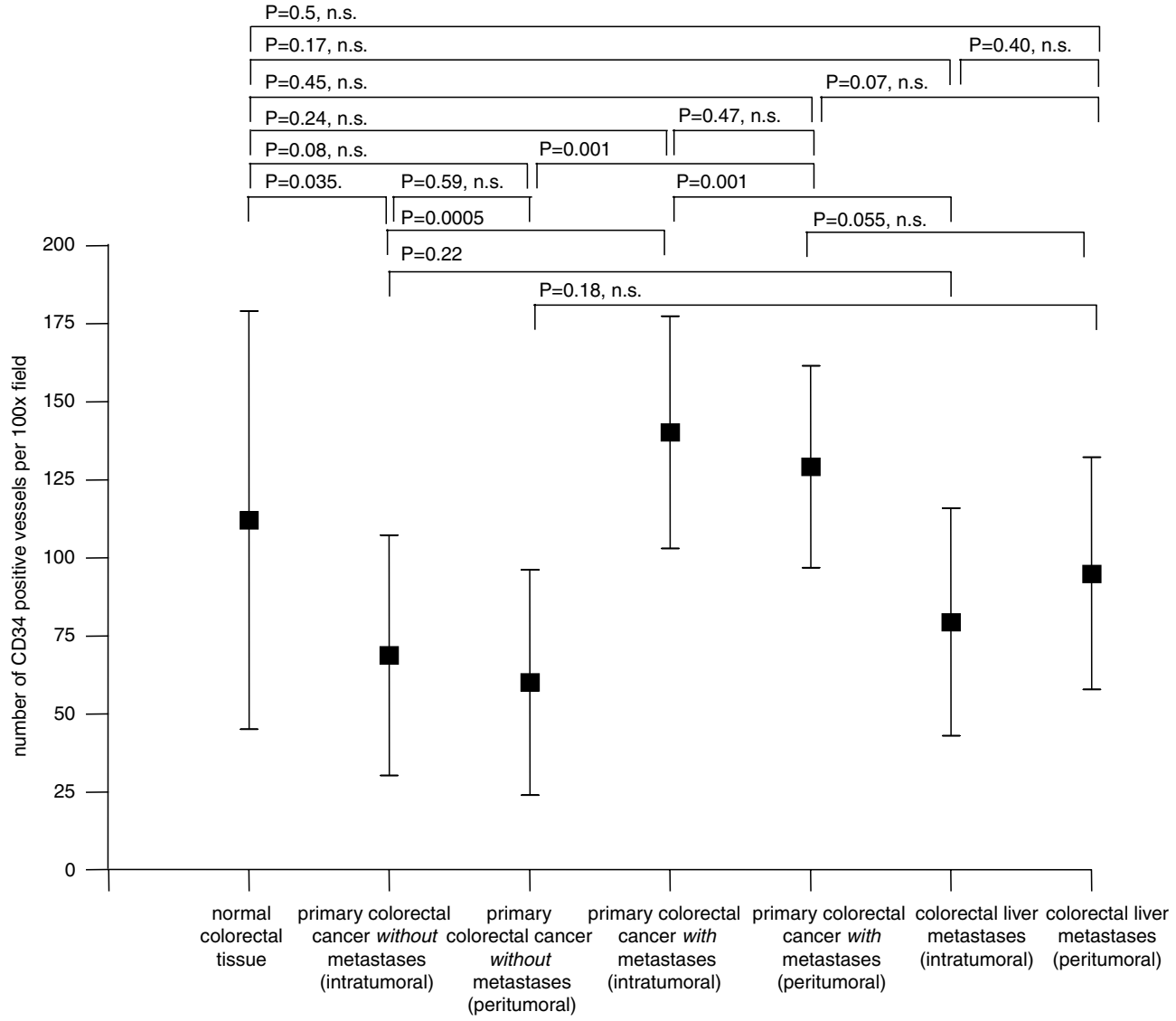

Figure 2 Intratumoral and peritumoral vascular density as determined by counting of CD34 stained vessels in normal colon, colorectal primary tumor without metastases, primary tumor with metastases and liver metastasis. Vascular density is expressed as number of vessels per $\times 100$ field, mean$\pm$s.d. (mean of the two counted fields for each specimen averaged for the 11 specimens in each group).

tumor (with and without metastases) and liver metastases, both within the tumor and along the peritumoral area, was evaluated by immunohistochemical analysis using CD45, the leukocyte common antigen.

The infiltration density of leukocytes (mean density$\pm$s.d, per $\times 100$ field) in normal colon was 832 ± 623 (Figure 4). In the intratumoral zone of colorectal primary tumors without metastases, the number of leukocytes was significantly lower (302 ± 208, $P=0.02$). Interestingly, in the peritumoral area of these lesions, where P-selectin expression was equal to the control group, leukocyte density was higher, and not differed significantly from the control group (463 ± 201 leukocytes/per $\times 100$ field). Moreover, both the intra- and peritumoral area of the colorectal primary tumor of patients with synchronous liver metastases were marked by an even further decrease in leukocyte counts. Again, these differences were more pronounced in the intratumoral area (122 ± 192) compared to the invasive front of the primary tumor (496 ± 369). Compared to patients without any lymph or liver metastases, these differences were significantly lower in the intratumoral area ($P=0.049$) of the tumor. Also, the liver metastases contained generally higher leukocyte count at the invasive front (477 ± 294) than in the intratumoral areas of these lesions (108 ± 243, $P=0.01$).

Taken together, the observed decrease in leukocyte infiltration in the peri- and intratumoral zone appeared to run parallel to the percentage of P-selectin-positive vessels.

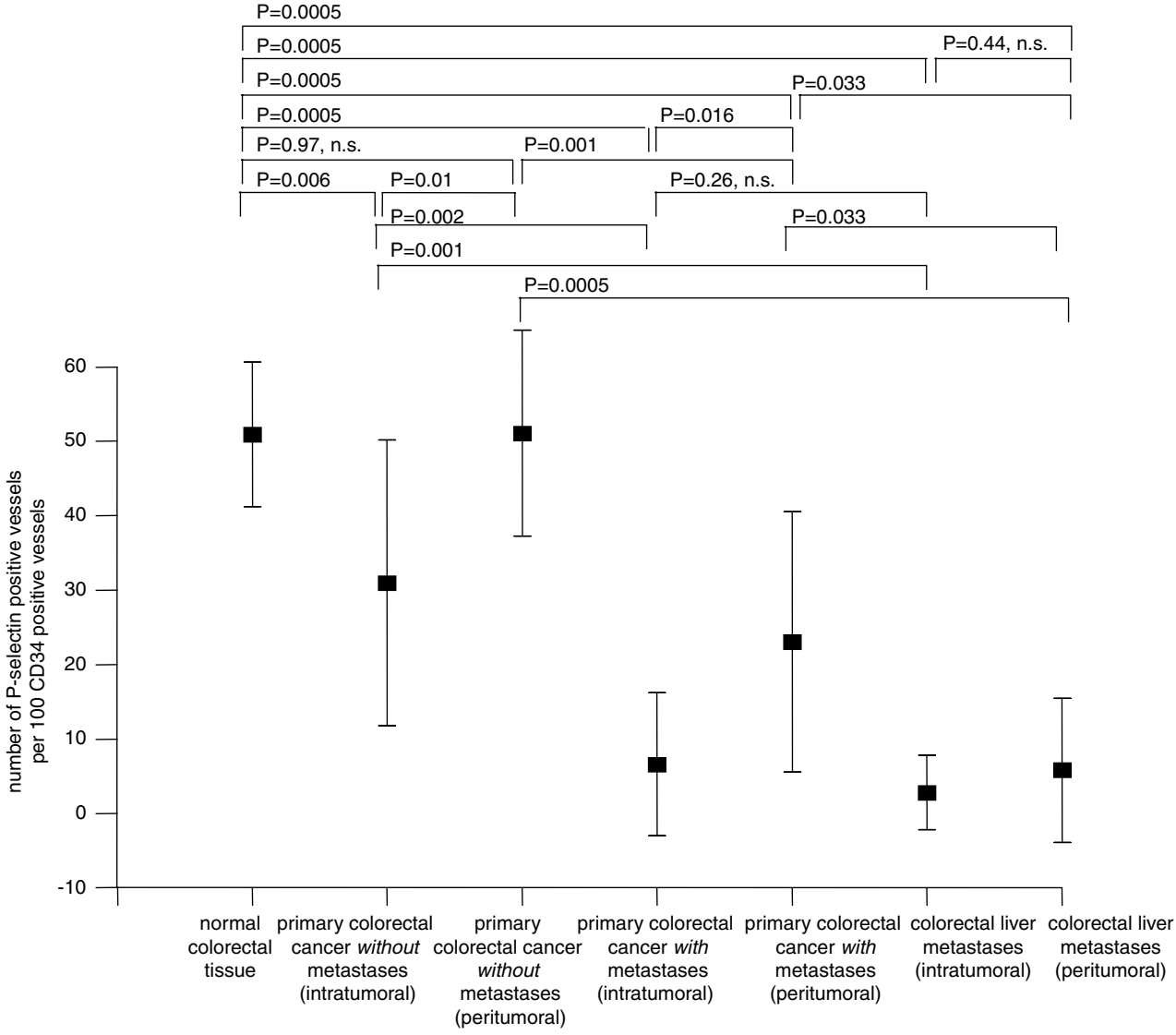

Figure 3 Intratumoral and peritumoral endothelial P-selectin expression in normal colon, primary colorectal tumor (without and with metastases) and liver metastases (number of P-selectin positive vessels per 100 CD34+ vessels per $\times 100$ field, mean$\pm$s.d.; mean of the two counted fields for each specimen averaged for the 11 specimens in each group). Note that levels of peritumoral and intratumoral P-selectin expression is reciprocal to the degree of progression in colorectal cancer.

Correlation analysis between the percentage of P-selectin-positive vessels and the number of leukocytes in the colorectal specimen series revealed a correlation of 0.44, representing a significant correlation ($P=0.0005$, Figure 5). A representative example of P-selectin expression and leukocyte colocalization is shown in Figure 1.

The infiltrate was quantitatively analyzed in more detail by counting the number of macrophages and granulocytes per $\times 100$ field.

The mean number$\pm$s.d. of macrophages per $\times 100$ field in all the 73 specimens analyzed was 223 ± 249. Macrophages were equally distributed in the different lesions and no differences between the groups could be demonstrated (data not shown). Granulocyte counts were highly variable both in normal and pathological tissues, and did not allow any conclusions (data not shown).

Discussion

The purpose of the study reported here was to evaluate the expression of P-selectin (CD62P) on the blood vessel endothelium in CRC. First, we analyzed the vascular density in all tissue specimens, to be able to express the number of P-selectin-positive vessels as a percentage of overall vessel density. Remarkably, a high vessel density was present in primary lesions that had already metastasized, confirming previous publications.[20] Our subsequent data showed a clear and progressive decrease in the

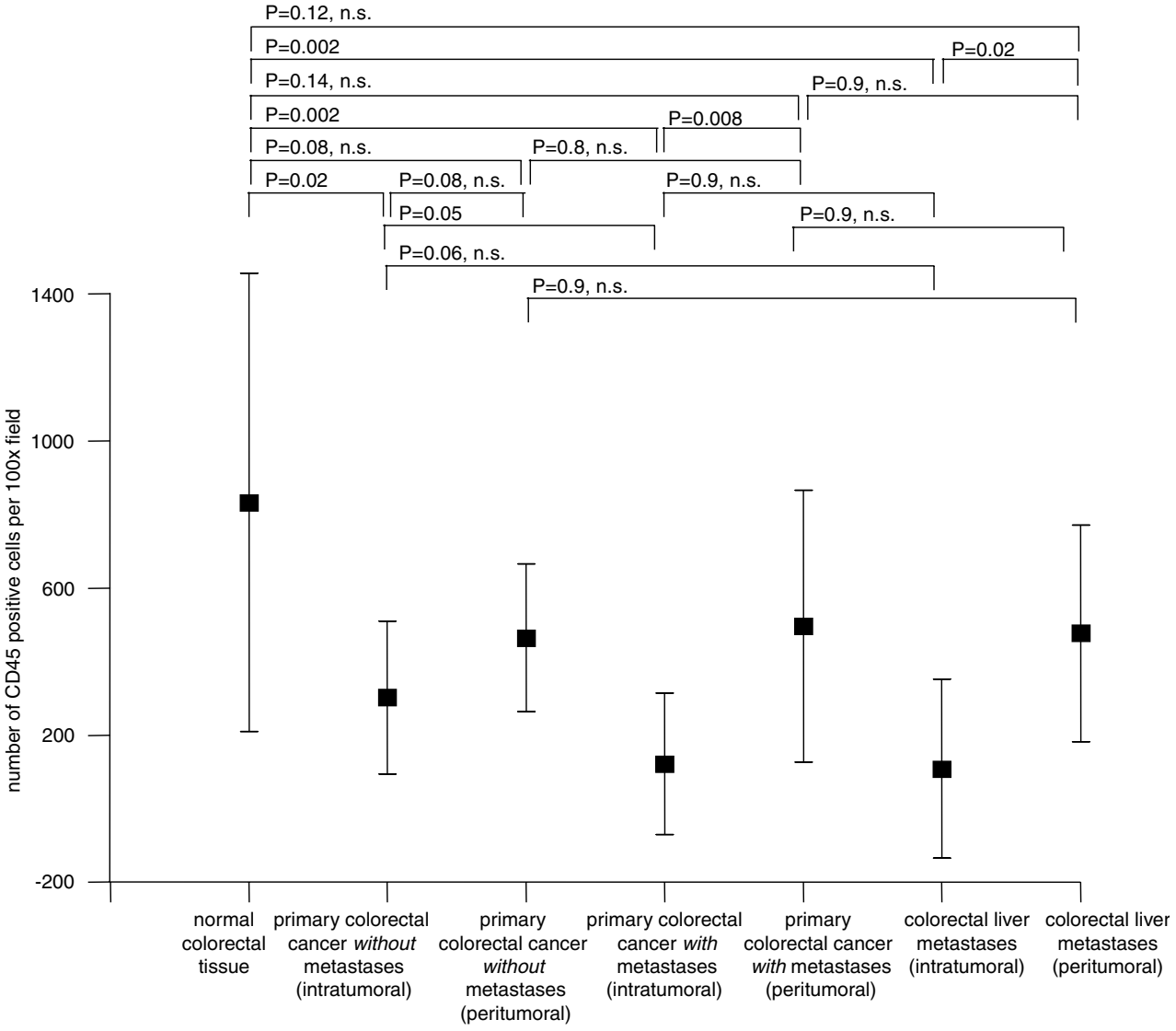

Figure 4 Intratumoral and peritumoral leukocyte density as assessed by CD45 staining of normal colon, primary tumor (without and with metastases) and liver metastases (number of leukocytes per $\times 100$ field, mean $\pm$ s.d.; mean of the two counted fields for each specimen averaged for the 11 specimens in each group). Note a dense distribution of leukocytes along the invasive margin of both the primary tumor and the metastases, whereas the intratumoral area is devoid of leukocytes.

number of P-selectin-positive blood vessels in a series of lesions ranging from normal colon, primary tumors without metastasis to the lymph nodes or liver to primary tumor specimens with comparable depths of invasion at the primary site; but with liver metastases, up to the liver metastases themselves. Similar to what we described earlier in cutaneous melanoma, we were able to demonstrate that the degree of vascular P-selectin expression is reciprocal to the degree of progression in CRC.[13]

Subsequent infiltrate analysis showed a close association between the expression level of P-selectin and the numbers of inflammatory cells. When comparing endothelial P-selectin expression in tissue section of patients without metastases, with lesions with the same invasion depth but with synchronous liver metastases, endothelial P-selectin expression was observed predominantly in the patients without metastases. Moreover, when comparing endothelial P-selectin expression in the invasive margin with that in the intratumoral area of both these types of lesions, a far more pronounced P-selectin expression along the invasive margin was observed. In the peritumoral margin, the most dense leukocyte infiltrates were also present. In contrast, inside the tumor lesion, a lesser percentage of blood vessels usually expressed this endothelial cell adhesion molecule, and fewer leukocytes had accumulated. We encountered these phenotypical variations not only in colorectal primary tumors but also in metastatic lesions which has, to the best of our knowledge, never been described before. Taken these observations together, the following conclusions may be drawn.

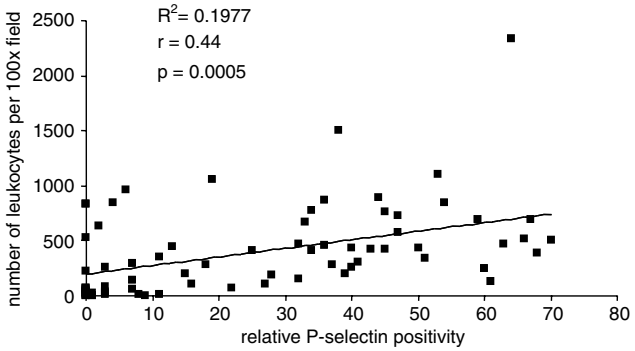

Figure 5 Correlation analysis between the percentage of P-selectin-positive vessels and the number of CD45+ leukocytes in the colorectal cancer tissue specimen series (normal colon, colorectal primary tumor (with and without metastases) and liver metastases). (Pearson's correlation coefficient: $r=0.44$, $P=0.0005$).

First, our data strongly suggest that there is heterogenecity of colorectal tumor vessels with regard to P-selectin expression: on peritumoral vessels, higher levels were observed. These phenotypical variations were encountered not only in the primary tumors but also in the metastatic lesions. In the peritumoral area of the metastases leukocyte infiltration, however, was more pronounced than that could be expected on the basis of the minor increase in endothelial P-selectin expression. We are currently analyzing the process of vessel formation and remodeling in colorectal liver metastases. Preliminary results demonstrate that in the peritumoral area, a conversion occurs from the well-organized hepatic sinusoids to an irregularly shaped vascular bed with an upregulation of several endothelial cell molecules, resembling the vasculature in the primary tumor. Therefore, we speculate that the process of leukocyte infiltration into the peritumoral area of liver metastases may be dependent on upregulation of endothelial adhesion receptors other than the selectins.

Next, compared to normal colorectal tissue, we found a downregulation in P-selectin expression and a concomitant decrease in leukocyte infiltration, both in the peritumoral and intratumoral area. Prevention of leukocyte infiltration into the tumor may represent a protective mechanism by which tumors escape immune surveillance. For example, leukocyte infiltration within tumors is generally associated with decreased tumor growth rate in carcinomas of the breast, stomach and colon.[18,21–23] Our results suggest that in CRC, malignant progression of the tumor leads to progressive P-selectin down regulation. This phenomenon has been described also for other endothelial cell-associated adhesion molecules in colorectal tumors (ICAM-1, E-selectin).[14]

Our results furthermore demonstrate that impaired leukocyte infiltration is not only associated with increased tumor growth, but also with the ability of tumor cells to metastasize. Since the primary tumors that had already metastasized, also had increased vascular density, it is difficult to dissect the mechanisms involved in these processes. Lack of P-selectin expression may result in deficient antitumor immunity, and thereby enhance growth of the primary tumor. On the other hand, a dense vascular bed will augment the tumor's blood supply and stimulate growth as well. It may, however, also facilitate tumor cell invasion and metastasis.

Previously, P-selectin expression and leukocyte infiltration was already related to adverse prognosis in patients with malignant melanoma.[24] It has been speculated that leukocytes secrete growth factors that stimulate tumor cell proliferation.[25] A downregulation in endothelial P-selectin expression may therefore result in the recruitment of fewer, growth factor producing, leukocytes and in a less growth stimulating microenvironment for the tumor cells.[26–28] However, our results demonstrate that, compared to patients with liver metastases, in patients without any metastases a high expression level of endothelial P-selectin was maintained, and that this is accompanied by high numbers of infiltrating leukocytes. As tumors without metastases generally have a less infiltrating character and a better prognosis, we suggest that the latter mechanism, that is, tumor growth promotion by infiltrating cells, is less likely to occur. Accordingly, Harrison *et al*[29] have demonstrated in patients with right-sided colon cancer, that lymphoid aggregates took precedence as independent prognostic factor for length of survival.

It has been demonstrated that macrophages play a key role in the induction of angiogenesis by the production of vascular endothelial growth factor (VEGF).[30] In the primary tumor, relatively high levels of endothelial P-selectin were expressed compared to the liver metastases. A decrease in infiltrating macrophages as a result of a downregulation of endothelial P-selectin could therefore explain the observed decrease in angiogenesis in the liver metastases. However, by analyzing the number of infiltrating macrophages, we found no differences between primary tumors and metastases, both intra- and peritumorally. Still, the lack of proliferation of the micovasculature in the metastases might be due to a decrease in P-selectin expression, but its mechanism remains unclear. We also speculated that the primary colorectal tumor produces anti-angiogenic factors, like angiostatin and endostatin, that inhibit angiogenesis and therefore reduce the number of vessels in its distant metastases, but this remains to be demonstrated in humans.

Taken together, we have demonstrated in a series of CRC lesions that the levels of vascular P-selectin expression are inversely related to the degree of tumor progression. Furthermore, leukocyte infiltration levels showed the same correlation in this series. Our results may have implications for cancer therapy in both the primary CRC and its metastases. Determining the levels of endothelial adhesion

molecules like P-selectin in tumors may have predictive value for the successfulness of immunotherapy. By influencing the adhesion characteristics of the intratumoral vessels, for example, selectin expression, via cytokine treatment, influx of immuno-competent cells may be encouraged.

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