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Serum endostatin levels are elevated in colorectal cancer and correlate with invasion and systemic inflammatory markers

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Background: Endostatin, a fragment of collagen XVIII, is an endogenous angiogenesis inhibitor with anti-tumour functions. However, elevated circulating endostatin concentrations have been found in several human cancers including colorectal cancer (CRC).

Methods: Serum endostatin levels were measured by enzyme-linked immunoassay from a series of 143 patients with CRC and from 84 controls, and correlated with detailed clinicopathological features of CRC, serum leukocyte differential count and C-reactive protein (CRP) levels.

Results: Patients with CRC had higher serum endostatin levels than the controls ($P = 0.005$), and high levels associated with age, tumour invasion through the muscularis propria and poor differentiation, but not with metastases. Endostatin levels showed a positive correlation with the markers of systemic inflammatory response and a negative correlation with the densities of tumour-infiltrating mast cells and dendritic cells. Collagen XVIII was expressed in tumour stroma most strikingly in blood vessels and capillaries, and in the muscle layer of the bowel wall.

Conclusions: Elevated endostatin levels in CRC correlate with systemic inflammation and invasion through the muscularis propria. Increased endostatin level may be a result of invasion-related cleavage of collagen XVIII expressed in the bowel wall. The negative correlations between serum endostatin and intratumoural mast cells and immature dendritic cells may reflect angiogenesis inhibition by endostatin.

Colorectal cancer (CRC) is one of the most common malignancies and among the leading causes of death in the industrialised world (Siegel *et al*, 2013). As in all solid tumours, angiogenesis is crucial for CRC growth, progression and metastasis, and tumours cannot grow beyond the size of few millimetres without angiogenesis (Folkman *et al*, 1963). In CRC, the control of normal physiologic angiogenesis inhibition is disrupted, allowing neovascularisation that supports tumour growth (Folkman and Klagsbrun, 1987; Jain, 2005).

Endostatin, one of the most potent inhibitors of angiogenesis, is a proteolytically cleaved 20-kDa C-terminal fragment of a vascular and epithelial basement membrane protein, collagen XVIII (O'Reilly *et al*, 1997). Endostatin inhibits angiogenesis via its ability to restrict endothelial cell proliferation (O'Reilly *et al*, 1997) and migration (Yamaguchi *et al*, 1999) and to induce endothelial cell apoptosis (Dhanabal *et al*, 1999). The antitumour effect of endostatin is not merely restricted to angiogenesis and endothelial cells, but it also directly inhibits tumour cell migration and

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invasion (Wilson *et al*, 2003; Folkman, 2006). Endostatin binds to several cell membrane proteins including $\alpha5\beta1$ and $\alpha v\beta3$ integrins (Rehn *et al*, 2001; Faye *et al*, 2009), glypicans (Karumanchi *et al*, 2001), and VEGF receptors 1, 2 and 3 (Kim *et al*, 2002; Kojima *et al*, 2008). It inhibits the activation of proMMP-2, pro-MMP-9 and proMMP-13 and the catalytic activity of MMP-2 and membrane type -1 MMP (Kim *et al*, 2000; Nyberg *et al*, 2003). The broad range of the molecular targets of endostatin suggests that it can affect the behaviour of the cells via numerous pathways. The extensive influence of endostatin on endothelial cells is based on its effect on gene expression: By using genome-wide microarray analysis, Abdollahi *et al*, 2004 showed that endostatin treatment of cultured human endothelial cells resulted in significant changes in 12% of the genes analysed.

Elevated circulating endostatin concentrations have been found in several human cancers. Despite the acknowledged antiangiogenic functions of endostatin, higher serum endostatin levels are associated with poor differentiation and advanced stage in CRC, gastric cancer and bladder cancer (Li *et al*, 2012; Szarvas *et al*, 2012) and with poor prognosis of the patient in bladder cancer, non-small cell lung cancer, gastric cancer and soft tissue sarcoma (Feldman *et al*, 2001; Suzuki *et al*, 2002; Woo *et al*, 2006; Szarvas *et al*, 2012). The effect of endostatin on endothelial cells depends on the length of exposure (Li *et al*, 2005), the type of endothelial cells (Schmidt *et al*, 2004) and the type of growth factor inducing endothelial cell proliferation (Delaney *et al*, 2006). Finally, the composition of the extracellular matrix with which the cells are in contact modifies the effect of endostatin (Delaney *et al*, 2006). Interestingly, endostatin has a tumour-specific optimal inhibition concentration, higher and lower dosages having less inhibitory effect (Celik *et al*, 2005; Tjin Tham Sjin *et al*, 2006). All in all, endostatin is associated with several fundamental aspects of cancer including tumour cell differentiation, cancer angiogenesis and lymphangiogenesis, and inflammatory cell infiltration (Brideau *et al*, 2007; Seppinen and Pihlajaniemi, 2011).

In this study, we aimed to enlighten the significance of serum endostatin levels in CRC patients. We measured systemic endostatin levels in 143 CRC patients and 84 healthy controls matched for age and gender and correlated the endostatin levels with clinicopathological parameters. Furthermore, in order to evaluate the contribution of both local and systemic inflammation to serum endostatin levels in CRC, we analysed the association of serum endostatin levels with local inflammatory cell densities in CRC tissue and with systemic inflammation as determined by C-reactive protein (CRP), blood leukocyte counts, neutrophil/lymphocyte ratio (NLR) and modified Glasgow prognostic score (mGPS) (Roxburgh and McMillan, 2010).

MATERIALS AND METHODS

Patients and sampling. All newly diagnosed CRC patients operated on in Oulu University Hospital between April 2006 and January 2010 ($n=344$) were introduced for this prospective study. Blood samples and surgical specimens were originally collected from 148 patients, who had signed an informed consent to participate and were eligible for the study. Thirty-two of the 148 patients (21.6%) received preoperative radiotherapy or chemoradiotherapy (RT/CRT) and were excluded from the inflammatory cell analyses because RT/CRT is a potential confounding factor affecting the local tumour characteristics and reducing inflammatory reaction of the tumours (Nagtegaal *et al*, 2002a). The RT/CRT control group was a stage-matched control group randomly selected from rectal cancer patients who had not received preoperative RT/CRT. Age- and sex-matched control serum samples were acquired from healthy voluntary blood donors (Finnish Red Cross, Oulu, Finland; $n=36$, age <65years) and cataract surgery patients (Oulu University Hospital; $n=50$, age $\geqslant65$years). The samples were centrifuged, and serum was stored at $-70\,^{\circ}C$ until further analysis. The study set-up is previously described (Kantola *et al*, 2012). From the blood samples, leukocyte differential count was utilised in the calculation of NLR, and CRP and serum albumin were utilised for the assessment of mGPS ($0=\text{CRP}\leqslant10\,\text{mg}\,\text{l}^{-1}$, $1=\text{CRP}>10\,\text{mg}\,\text{l}^{-1}$, and $2=\text{CRP}>10\,\text{mg}\,\text{l}^{-1}$ and albumin $<35\,\text{g}\,\text{l}^{-1}$). (Roxburgh and McMillan, 2010). The blood NLR and mGPS are systemic inflammatory markers found to have significant prognostic value in CRC (Roxburgh and McMillan, 2010).

Collagen XVIII and inflammatory cell immunohistochemistry. Immunohistochemical analyses of collagen XVIII (rabbit polyclonal antibody QH48) and inflammatory cell markers were carried out on formalin-fixed paraffin-embedded 3.5-μm sections as described earlier (Saarela *et al*, 1998; Väyrynen *et al*, 2013). The inflammatory cell markers used in this study were CD3 (T cells), CD8 (cytotoxic T cells), FoxP3 (regulatory T cells), CD68 (monocyte-macrophage lineage cells), neutrophil elastase (neutrophilic granulocytes), mast cell tryptase (mast cells), CD83 (mature dendritic cells (DCs)) and CD1a (immature DCs) (Väyrynen *et al*, 2013). Bound antibodies were detected using peroxidase-based EnVision kit (Dako, Copenhagen, Denmark). 3,3'-Diaminobenzidine was used as the chromogen and all sections were counterstained with haematoxylin. Cells were quantitated by using image analysis and the quantity of blood vessels surrounded by collagen XVIII-expressing basement membrane was evaluated on a 4-tiered scale from the captured images of the invasive front and the tumour stroma (Väyrynen *et al*, 2012, 2013).

Serum endostatin assays. Endostatin concentrations were measured from serum samples of 148 CRC patients and 84 age- and sex-matched controls. Serum endostatin levels were determined by using the commercial Quantikine Human Endostatin Immunoassay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Previously produced recombinant human endostatin (Rehn *et al*, 2001) was used as a control in the assay. Colour intensity of the samples was measured with a Victor3 plate reader (PerkinElmer, Waltham, MA, USA). All the assays were performed in duplicate and the mean values were used as the final concentration. Finally, endostatin measures of 143 CRC patients, 113 CRC patients without RT/CRT and 84 controls were included for the analyses. One of the samples was ignored because of having an endostatin value above the standard curve and four samples were left aside because the measured duplicates differed from each other by at least 20%.

Follow-up. All CRC patients who underwent surgery were followed up for tumour recurrence at regular intervals for up to 5 years. For disease-free survival (DFS) analyses, the time to the end point was calculated from the date of diagnosis of CRC until the date of locoregional or systemic CRC recurrence. The DFS analysis included 81.4% (92 out of 113) of the patients, while excluded patients (18.6%, 21 out of 113) underwent palliative operation. The median follow-up time was 51.5 months (range 0.1–60 months). For cancer-specific survival (CSS) and overall survival (OS) analyses, all 113 patients were included, with a median follow-up of 57.9 months (range 0.1–60 months). The OS for all patients was 65.5% (74 out of 113).

Statistical analysis. Normally distributed continuous variables are presented as mean (standard deviation, s.d.), whereas other continuous variables are presented as median (interquartile range). IBM SPSS Statistics 19 was used for statistical analysis (IBM, Chicago, IL, USA). Statistical significances of the differences in serum endostatin levels between the different study groups and age, gender, stage, grade and tumour location categories were

analysed by Mann–Whitney *U*-test or Kruskal–Wallis test. Univariate correlations are presented as Pearson correlation coefficients. A multiple linear regression analysis using stepwise method was performed to analyse the independent association of serum endostatin to the clinicopathological features of the cancer. Kaplan–Meier curves were used to visualise the differences of DFS, CSS and OS for patient groups stratified based on serum endostatin levels, and differences between groups were evaluated by the log rank test. The serum endostatin cutoff value (172 ng ml^{-1}) for survival analyses was obtained from receiver operating characteristics (ROC) analysis, in which optimal cutoff scores for serum endostatin levels in discriminating CRC patients from healthy controls were defined. In all the tests, a *P*-value less than 0.05 was considered statistically significant.

RESULTS

Demographic characteristics and serum endostatin in CRC. The characteristics of CRC patients and healthy controls are presented in Table 1 and the preoperative serum endostatin levels in Table 2. Because radiation is known to induce microvascular damage and changes in microvascular density (Seemann *et al*, 2012) as well as to cause a pronounced fibroblastic reaction (Nagtegaal *et al*, 2002b), we first evaluated the effect of preoperative RT/CRT on endostatin levels of CRC patients. For this, we divided the CRC patient group into three subgroups (Table 1). The patients receiving RT/CRT had similar endostatin levels to patients not receiving RT/CRT and matched for tumour stage and location (RT/CRT control group) (Table 2; median 154.1 ng ml^{-1} *vs* 150.3 ng ml^{-1}, $P = 0.833$). Although preoperative RT/CRT did not affect serum endostatin levels, we excluded the RT/CRT group from the subsequent analyses because of the known effects of RT/CRT on local characteristics of the tumour (Nagtegaal *et al*, 2002a; Seemann *et al*, 2012).

Serum endostatin levels were significantly increased in CRC patients without RT/CRT compared with healthy controls (Figure 1A; Table 2; median 151.1 *vs* 136.1, $P = 0.005$). A ROC analysis was conducted to test the feasibility of serum endostatin in discriminating CRC patients without preoperative RT/CRT from healthy controls. It yielded an area under the curve (AUC) of 0.618 (95% confidence interval 0.539–0.696). Using a cutoff value of 172 ng ml^{-1}, discriminating specificity was 0.655 and sensitivity 0.798.

Endostatin serum levels were similar in females and males (Table 3). The endostatin levels were significantly higher in elderly patients ($\geqslant$65 years) compared with younger ($<$65 years) patients ($P = 0.014$). In the controls, the effect of age on serum endostatin concentration was similar and even more distinct ($P = 7.4$ E-10).

Serum endostatin levels in relation to clinicopathological parameters. The relationships between serum endostatin levels and clinicopathological variables of CRC are presented in Table 3. TNM stage I patients had significantly lower serum endostatin levels compared with more advanced stages ($P = 0.014$). Deeper local invasion (T) was associated with a trend towards higher serum levels of endostatin ($P = 0.055$), and invasion through the muscularis propria (T1-2 *vs* T3-4) was associated with higher serum endostatin concentrations ($P = 0.007$; Table 3; Figure 1B). The presence of regional ($P = 0.802$) or distant ($P = 0.790$) metastases did not have a significant effect on serum endostatin concentrations. WHO grade 3 tumours showed a tendency towards higher endostatin levels compared with grade 1 or 2 tumours

Table 1. Characteristics of the patients with CRC and the controls

	All CRC patients ($n = 143$)	CRC patients without RT/CRT ($n = 113$)	CRC patients with RT/CRT ($n = 30$)	CRC patients RT/CRT control group[a] ($n = 31$)	Healthy controls ($n = 84$)
Age, mean (s.d.)	67.0 (11.3)	68.0 (11.3)	63.5 (10.7)	68.1 (10.4)	66.9 (10.3)
Gender					
Male	77 (53.8%)	56 (49.6%)	21 (70%)	20 (64.5%)	44 (52.4%)
Female	66 (46.2%)	57 (50.4%)	9 (30%)	11 (35.5%)	40 (47.6%)
Preoperative RT/CRT					
Yes	30 (21%)	0 (0%)	30 (100%)	0 (0%)	
No	113 (79%)	113 (100%)	0 (0%)	31 (100%)	
Tumour location					
Proximal colon	48 (33.6%)	48 (42.5%)	0 (0%)	0 (0%)	
Distal colon	27 (18.9%)	27 (23.9%)	0 (0%)	0 (0%)	
Rectum	68 (47.6%)	38 (33.6%)	30 (100%)	31 (100%)	
WHO grade					
Grade 1	20 (14%)	15 (13.3%)	5 (16.7%)	5 (16.1%)	
Grade 2	105 (73.4%)	84 (74.3%)	21 (70%)	24 (77.4%)	
Grade 3	18 (12.6%)	14 (12.4%)	4 (13.3%)	2 (6.5%)	
TNM stage					
Stage I	25 (17.6%)	18 (16.1%)	7 (23.3%)	8 (25.8%)	
Stage II	54 (38.0%)	45 (40.2%)	9 (30%)	8 (25.8%)	
Stage III	45 (31.7%)	31 (27.7%)	14 (46.7%)	14 (45.2%)	
Stage IV	18 (12.7%)	18 (16.1%)	0 (0%)	1 (3.2%)	

Abbreviations: CRC = colorectal cancer; RT/CRT = radiotherapy or chemoradiotherapy; s.d. = standard deviation; TNM = tumour, node, metastasis; WHO = World Health Organization.
[a]Without RT/CRT.

($P=0.055$). The location of the tumour did not have a significant effect on serum endostatin levels ($P=0.162$). Tissue remodelling following necrosis could potentially release endostatin from its parent molecule, extracellular collagen XVIII. Thus, we analysed the possible association of serum endostatin level with the extent of necrosis, but no correlation was found ($P=0.769$).

Survival analysis. To assess the prognostic significance of serum endostatin level, patients were divided into high (<172 ng ml^{-1}) and low serum endostatin ($\geqslant 172$ ng ml^{-1}) groups, and a Kaplan–Meier survival analysis was carried out. The adequacy of the cutoff point was verified with ROC analysis (data not shown). The two-tiered classification of serum endostatin levels did not significantly associate with DFS (76.2% *vs* 89.6%; $P=0.185$; Figure 2A), CSS (74.3% *vs* 66.7%; $P=0.455$; Figure 2B) or OS (70.3% *vs* 56.4%; $P=0.111$; Figure 2C).

Correlation between serum endostatin and local immune cell infiltration. Endostatin has been suggested to modulate inflammatory reactions and tumour infiltration of leukocytes (Brideau *et al*, 2007), and thus we evaluated the associations between serum endostatin levels and local inflammatory cell densities in CRC tissue (Table 4). Of the studied inflammatory cells, CD1a$^+$ DCs in tumour stroma, CD83$^+$ DCs at the invasive front and mast cells in tumour stroma showed a negative correlation with serum endostatin levels.

Correlation of serum endostatin and systemic inflammation markers. We evaluated the correlations between serum endostatin levels, blood leukocyte counts and CRP concentration in patients with CRC. Of the studied parameters, CRP levels, NLR, total leukocyte count and neutrophil count showed a positive correlation with the serum endostatin levels (Table 5), and endostatin levels were higher in patients with moderate or high mGPS score as compared with those with low mGPS (Table 6).

Multivariate analyses. Next, a multiple linear regression analysis was performed to analyse the independent associations between serum endostatin levels and clinicopathological features of the cancer as well as immune cell infiltrates and systemic inflammatory cell counts. The variables analysed included age, gender, BMI, tumour location, distant metastases, nodal metastases, invasion through the muscularis propria, tumour-destructing peritumoural inflammatory infiltrate and necrosis. Of these factors, the

Table 2. Serum endostatin levels in CRC patients compared with healthy controls

	N	Endostatin (serum) ng ml^{-1}, median (IQR)	*P*-value
Healthy controls	84	136.1 (109.0–166.4)	
All CRC patients	143	151.1 (125.5–194.1)	*vs* healthy controls, $P=$ **0.004**
CRC patients without RT/CRT	113	151.1 (125.6–195.4)	*vs* healthy controls, $P=$ **0.005**
CRC patients with RT/CRT	30	154.1 (117.5–189.0)	*vs* healthy controls, $P=0.102$
RT/CRT control group	31	150.3 (131.0–174.4)	*vs* patients with RT/CRT, $P=0.833$

Abbreviations: CRC = colorectal cancer; RT/CRT = radiotherapy or chemoradiotherapy; IQR = interquartile range. The *P*-values are for Mann–Whitney test.

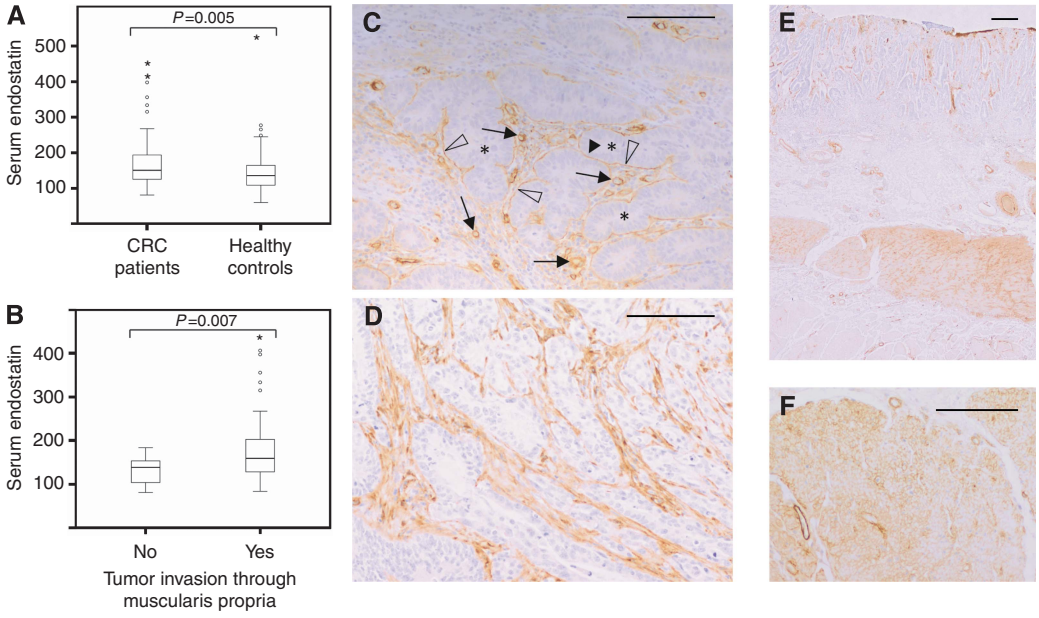

Figure 1. Serum endostatin in colorectal cancer and association with primary tumour invasion. (**A**) CRC patients had higher serum endostatin levels than age- and sex-matched healthy controls. (**B**) Tumour invasion through the muscularis propria associated with higher serum endostatin concentrations. (**C–F**) Collagen XVIII immunohistochemistry. (**C**) Blood vessels and capillary structures (arrows) show strong collagen XVIII expression, whereas no explicit staining could be found in carcinoma cells (asterisks). Collagen XVIII localised into the basement membranes surrounding the invasive tumour cell islets in some CRC cases (arrowheads). (**D**) In some CRC cases, collagen XVIII localised around myofibroblasts in desmoplastic tumour stroma. (**E**) In the bowel wall smooth muscle, collagen XVIII was detected in the muscle layer. (**F**) Higher magnification reveals collagen XVIII expression between muscle cells corresponding the location of basal laminae. Scale bars: c,d,f, 100 μm, e, 200 μm.

Table 3. Serum endostatin levels in relation to clinical and pathological characteristics of CRCs

	Endostatin ng ml^{-1}, median (IQR)	*P*-value
Gender		
Male patients	148.7 (122.0–183.3)	0.503
Female patients	154.2 (125.6–201.9)	
Male controls	121.4 (107.1–166.9)	0.205
Female controls	146.5 (113.9–166.4)	
Age		
Patients < 65 years ($n = 40$)	138.6 (105.3–170.4)	**0.014**
Patients ⩾ 65 years ($n = 73$)	159.7 (135.7–201.3)	
Controls < 65 years ($n = 36$)	108.1 (101.2–127.0)	**7.4 E-10**
Controls ⩾ 65 years ($n = 48$)	154.2 (137.7–179.1)	
TNM Stage		
Stage I ($n = 18$)	132.6 (103.1–149.8)	**0.014**
Stage II ($n = 45$)	164.4 (135.7–222.0)	
Stage III ($n = 31$)	149.0 (125.7–173.4)	
Stage IV ($n = 18$)	159.7 (108.9–211.3)	
TNM classes T1-T4		
T1 ($n = 5$)	147.9 (109.4–156.2)	0.055
T2 ($n = 17$)	136.2 (103.9–154.1)	
T3 ($n = 82$)	159.5 (130.2–204.6)	
T4 ($n = 9$)	172.7 (98.2–195.4)	
TNM classes T1-T2 *vs* T3-T4		
T1-T2 ($n = 22$)	139.1 (104.1–154.1)	**0.007**
T3-T4 ($n = 91$)	159.7 (127.7–203.9)	
TNM classes N0-N2		
N0 ($n = 67$)	150.3 (125.1–198.9)	0.802
N1 ($n = 26$)	153.6 (118.8–173.7)	
N2 ($n = 19$)	149.0 (138.1–203.9)	
TNM classes M0-M1		
M0 ($n = 95$)	150.3 (127.0–190.2)	0.790
M1 ($n = 18$)	159.7 (108.9–211.3)	
WHO Grade 1–3		
Grade 1 ($n = 15$)	125.1 (106.9–179.4)	0.064
Grade 2 ($n = 84$)	152.1 (128.4–188.7)	
Grade 3 ($n = 14$)	180.4 (133.9–279.8)	
WHO Grade 1–2/3		
Grade 1–2 ($n = 99$)	149.0 (125.1–184.2)	0.055
Grade 3 ($n = 14$)	180.4 (133.9–279.8)	
Tumour location		
Proximal colon ($n = 48$)	160.3 (130.0–212.1)	0.162
Distal colon ($n = 27$)	154.1 (115.1–172.7)	
Rectum ($n = 38$)	150.0 (124.1–176.9)	
Necrosis		
None or rare ($n = 67$)	149.0 (118.4–184.2)	0.769
Frequent small ($n = 29$)	154.1 (131.5–195.4)	
Broad ($n = 17$)	153.1 (109.3–212.7)	

Abbreviations: CRC = colorectal cancer; IQR = interquartile range; TNM = tumour, node, metastasis; WHO = World Health Organization. The *P*-values are for Mann–Whitney or Kruskal–Wallis test.

regression analysis using stepwise method found high age, invasion through the muscularis propria and poor differentiation as the three most important predictors of serum endostatin levels (Table 7), with the ability to explain 43.3% of the variability in serum endostatin levels.

In a second model, in addition to the variables above, we included the inflammatory markers, that is, the counts of stromal mast cells, peritumoural mature DCs, stromal immature DCs, serum CRP, and blood leukocytes, lymphocytes, neutrophils and NLR, which showed correlation with serum endostatin in univariate analysis. This analysis indicated that blood NLR and patient age were positively and stromal immature DC count negatively associated with serum endostatin.

Collagen XVIII immunohistochemistry. We used immunohistochemistry to assess the expression patterns of collagen XVIII, the precursor molecule of endostatin. In CRC specimens, collagen XVIII expression mainly localised to the endothelial cells of the blood vessels (Figure 1C). No explicit positivity could be found in carcinoma cells. Collagen XVIII also localised into basement membrane structures surrounding invasive tumour cell islets (Figure 1C) and around myofibroblasts in some desmoplastic tumour stroma areas (Figure 1D). We graded the quantity of positive blood vessels at tumour stroma and invasive front but found no correlation with systemic endostatin levels (data not shown). In the bowel wall, collagen XVIII was most strikingly expressed in the muscle layer between muscle cells corresponding to the location of basal laminae structures (Figure 1E and F).

DISCUSSION

Angiogenesis regulatory proteins are important modifiers of tumour growth and invasion, representing potential biomarkers for diagnostic and prognostic assessment and potential targets for CRC therapy. There is also a linkage between tumour-associated inflammatory reactions and tumour angiogenesis (Mantovani *et al*, 2008). Thus, it would be of importance to understand the interactions and mutual regulation of these complex systems. In this study, serum endostatin levels in CRC were first analysed in relation to major clinicopathological parameters. Second, the serum endostatin levels were correlated with blood leukocyte counts and inflammatory cell densities in CRC tissue.

We found that preoperative serum levels of endostatin are increased in CRC patients compared with healthy controls. A similar association has been reported in several human cancers and recently also in gastrointestinal cancers, including CRC, gastric cancer and hepatocellular cancer (Li *et al*, 2012). It has also been shown that the removal of a primary colorectal tumour leads to a decrease in serum endostatin levels (Wu *et al*, 2004; Peeters *et al*, 2005). However, our study suggests that endostatin is unlikely to prove to be a valuable tool in CRC diagnosis or follow-up, because of relatively low AUC (0.618) in discriminating the patients from healthy controls in ROC analysis. Especially, the elderly appeared to have high serum endostatin levels in the absence of CRC. The mechanism of such age-related increase in endostatin levels is unknown, but it might be associated with age-related increase in cardiovascular disease and the elevated endostatin levels in these patients (Mitsuma *et al*, 2007; Carlsson *et al*, 2013).

Li *et al*, 2012 have reported that increased serum endostatin levels correlate with CRC progression. Our results also showed a trend towards elevated serum endostatin levels in the presence of deeper local invasion of the tumour (T classification) in univariate analysis (Table 3; $P = 0.055$) and significant correlation between higher serum endostatin levels and the invasion of the cancer tissue through the muscularis propria (T1-2 *vs* T3-4) in univariate (Table 3; $P = 0.007$) and multivariate analysis (Table 7; $P = 0.032$).

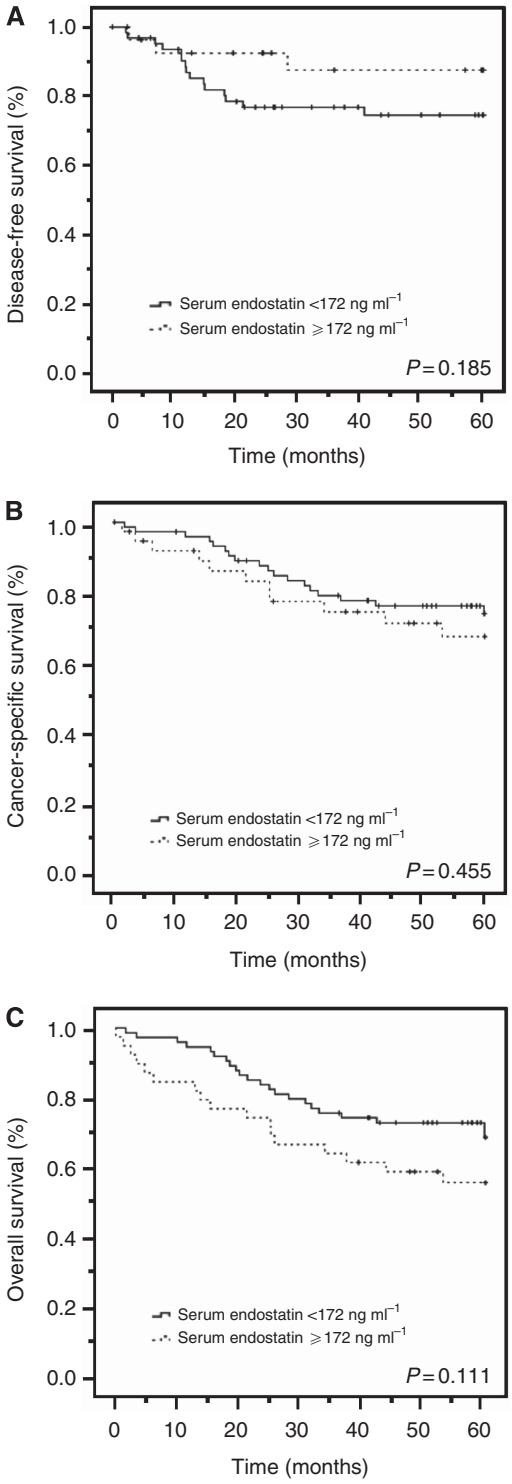

Figure 2. Kaplan–Meier survival analyses of patients with CRC. (**A**) Serum endostatin level had no effect on disease-free survival, (**B**) cancer-specific survival or (**C**) overall survival.

The presence of metastases did not further increase the serum endostatin levels in our study.

Immunohistochemical analysis indicated that the CRC cells do not express collagen XVIII, the source of endostatin, whereas blood vessels and capillary structures showed strong collagen XVIII positivity (Figure 1C). In some tumour areas, collagen XVIII also localised around myofibroblastic stromal cells and basement membrane structures encircling invasive tumour cell islets. In the bowel wall, collagen XVIII expression was most prominent around smooth muscle cells of the muscle layer (Figure 1E). This suggests that the increase of serum endostatin levels may result from the degradation of collagen XVIII and the release of endostatin, when

Table 4. Correlation of serum endostatin levels with local areal density of inflammatory cells in CRC specimens

	Pearson r	*P*-value
Invasive front		
CD3	−0.004	0.966
CD8	0.074	0.434
FoxP3	−0.023	0.810
CD68	0.132	0.164
CD83	−0.189	**0.047**
CD1a	−0.160	0.090
Mast cell tryptase	−0.103	0.276
Neutrophil elastase	0.084	0.378
Tumour stroma		
CD3	0.059	0.537
CD8	0.159	0.092
FoxP3	0.095	0.317
CD68	0.023	0.805
CD83	−0.140	0.145
CD1a	−0.281	**0.003**
Mast cell tryptase	−0.214	**0.023**
Neutrophil elastase	2.2E-4	0.998
Intraepithelial		
CD3	0.038	0.690
CD8	0.042	0.671

Abbreviation: CRC = colorectal cancer. Numbers indicate Pearson correlation coefficients (r) for logarithmically transformed variables.

Table 5. Correlations between serum endostatin and CRP concentrations, and peripheral blood white blood cell counts and the NLR

	Endostatin Pearson r	*P*-value
CRP	0.274	**0.003**
NLR	0.283	**0.003**
Leukocytes	0.199	**0.035**
Lymphocytes	−0.074	0.436
Monocytes	0.144	0.130
Neutrophils	0.298	**0.001**

Abbreviations: CRP = C-reactive protein; NLR = neutrophil/lymphocyte ratio. Numbers indicate Pearson correlation coefficients (r) for logarithmically transformed variables.

Table 6. Difference in endostatin levels within mGPS

	Endostatin ng ml^{-1}, median (IQR)	*P*-value
mGPS		
0 (n = 88)	148.2 (121.3–178.1)	
1–2 (n = 25)	180.4 (141.7–222.0)	**0.017**

Abbreviations: IQR = interquartile range; mGPS = modified Glasgow prognostic score. *P*-value is for Mann–Whitney test.

Table 7. Multiple linear regression model of serum endostatin level in colorectal cancer patients

Independent	Beta	*P*-value
Model 1		
Age	0.291	**0.001**
Grade	0.239	**0.008**
Invasion through muscularis propria	0.192	**0.032**
Model 2		
Blood NLR	0.231	**0.013**
Age	0.298	**0.001**
$CD1a^+$ cell count, stromal	−0.282	**0.002**

Abbreviation: NLR = neutrophil/lymphocyte ratio.

cancer cells invade through the muscle layers of the bowel wall. Although the serum endostatin levels positively correlated with T classification, our results indicated that there is no significant association between serum endostatin levels and survival.

Endostatin can be cleaved from collagen XVIII by several proteinases, such as MMPs-3, -7, -9, -13, -14 and -20, elastase and cathepsin L (Seppinen and Pihlajaniemi, 2011). Both tumour cells and tumour-associated local inflammatory cells are able to produce several of these enzymes (Nielsen *et al*, 1996; Brabletz *et al*, 1999; Heljasvaara *et al*, 2005; González *et al*, 2007). In accordance with an earlier report (Li *et al*, 2012), we found a significant correlation between high serum endostatin concentration and poor tumour differentiation. The expression of MMPs-7, -9, -13 and -14 have been reported to correlate with poor differentiation of CRC (Zhang *et al*, 2012; Yang *et al*, 2012, 2013; Bi *et al*, 2013), potentially contributing to increased serum endostatin levels in the poorly differentiated tumours. Although the correlation between the numbers of tumour infiltrating inflammatory cells and serum endostatin levels were generally weak, subpopulations of inflammatory cells may also represent a source of collagen XVIII-degrading enzymes.

We evaluated the relationships between serum endostatin levels and local inflammatory cell infiltration in CRC tissue. Interestingly, we found a negative correlation between serum endostatin levels and the numbers of mast cells and immature DCs in tumour stroma and mature DCs at the invasive front. Our earlier study (Väyrynen *et al*, 2013) showed that $CD1a^+$ immature DC and mast cell counts, unlike T cell and $CD83^+$ mature DC counts, do not associate with stage, and these two cell types clustered far apart from other inflammatory cells in the hierarchical clustering, suggesting that these cell types are less relevant in tumour-associated immune responses. Instead, it has been reported that immature DCs and especially mast cells promote angiogenesis in certain conditions (Huang *et al*, 1994; Maltby *et al*, 2009; Fainaru *et al*, 2010).

The limitation of our study was the lack of experimental data. Therefore, it is not possible to conclude convincingly whether the observed negative correlations between serum endostatin levels and the numbers of tumour-infiltrating mast cells and DCs (1) reflect higher endostatin concentrations inhibiting angiogenesis, being related to a decreased number of angiogenesis-related cell types, (2) signify a direct inhibitory effect of endostatin on mast cells and DCs or (3) result from some other unconsidered factor. Moreover, a potential decrease in microvessel number or perimeter caused by endostatin could explain the decreased immune cell infiltration in the tumours (Vlems *et al*, 2004). However, our results suggest a specific effect on mast cell and DC infiltration, because the densities of other tumour-infiltrating leukocytes did not correlate with serum endostatin levels.

The effect of endostatin on mast cells has earlier been reported by Brideau *et al*, 2007 using a carcinogen-induced skin tumourigenesis model in J4 mice overexpressing endostatin in their keratinocytes. Elevated endostatin levels in J4 mice reduced the number of VEGF-C-producing mast cells in the tumour tissue, in addition to which endostatin inhibited mast cell migration and adhesion *in vitro*. Brideau *et al*, 2007 also detected reduced numbers of peritumoural lymphatic vessels in J4 mice, suggesting an inhibitory effect of endostatin on lymphangiogenesis, at least partially resulting from the effect of endostatin on VEGF-C-expressing mast cells. Potential mechanisms of the direct interactions between mast cell and endostatin remain hypothetical. Potentially, endostatin may interact with integrins $\alpha5\beta1$ and $\alpha v\beta3$, expressed by both mast cells (Columbo *et al*, 1995; Columbo and Bochner, 2001) and DCs (Jancic *et al*, 1998; Skoberne *et al*, 2005), which also serve as binding partners for endostatin in endothelial cells (Rehn *et al*, 2001).

In patients with CRC, we detected positive correlations between serum endostatin and markers of systemic inflammation, including serum CRP, blood NLR and mGPS. In recent years, it has become apparent, that systemic inflammation in patients with cancer predicts poor outcome independently of tumour stage (McMillan, 2013). The relationships between serum endostatin and systemic inflammation have not been studied in patients with cancer, and the mechanisms linking endostatin and systemic inflammation are unknown. As discussed below, the numbers of tumour-associated leukocytes did not show correlation with endostatin levels (Table 4). This suggests that other mechanisms are involved, such as products released to the circulation as cancer cells invade.

In conclusion, our results suggest that elevated endostatin levels in CRC may be released by invading cancer cells cleaving endostatin from collagen XVIII. Endostatin levels correlated with markers of systemic inflammation but the mechanisms remain speculative. There were negative correlations between serum endostatin levels and the numbers of intratumoural mast cells and DCs, which could reflect higher endostatin concentrations inhibiting angiogenesis, being related to a decreased number of angiogenesis-related cell types, or signifying a direct inhibitory effect of endostatin on mast cells and DCs.

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