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Angiogenic cells, macroparticles and RNA transcripts in laparoscopic vs. open surgery for colorectal cancer

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Key words: colorectal cancer, laparoscopic/open colectomy, angiogenesis, endothelial cells, growth factors

Abbreviations: CECs, circulating endothelial cells; CEPs, circulating endothelial progenitors; VEGFR-2, -3, vascular endothelial growth factor receptor 2, 3; VEGF-A, -B, -C, -D, vascular endothelial growth factor A, B, C, D; VE-Cadherin, vascular endothelial cadherin; LC, laparoscopic colectomy; MP, macroparticles; OC, open colectomy; PDGFR-β, platelet derived growth factor receptor β

Background: Angiogenesis is crucial for tissue repair and cancer progression. We investigated a panel of angiogenic cells, macroparticles and RNA transcripts before, during and after laparoscopic colectomy or open colectomy for colorectal cancer.

Results: Viable and apoptotic circulating endothelial cells were significantly increased after open but not after laparoscopic colectomy ($p < 0.01$). A significant decrease of circulating mRNA coding for VEGFR-C and D and PDGFR-β was found after laparoscopic but not after open colectomy.

Methods: A total of 24 patients were enrolled. Viable and apoptotic circulating endothelial cells, progenitors and macroparticles were evaluated by flow cytometry. The number of copies of angiogenesis-related RNA transcripts were evaluated by quantitative PCR.

Conclusion: Open, but not laparoscopic colectomy, was associated with a significant post-operative increase in circulating endothelial cells, either apoptotic (likely due to surgery-related vascular damage) and viable (likely representing vascular remodeling). Circulating RNA copies coding for some angiogenic genes were significantly decreased after laparoscopic colectomy likely because of the removal of the tumor lesion. This decrease was not observed after open colectomy, were a more pronounced wave of angiogenesis related to wound healing was expected. These results indicate a relevant wave of angiogenesis-related cells and transcripts after open but not after laparoscopic colectomy.

Introduction

Laparoscopic colectomy (LC) was first reported almost 20 years ago.[1] At its beginning, LC was not widely accepted for malignancy because of concerns about the radicality of the resection, the oncologic outcomes and the high incidence of wound recurrence.[2,3] Nonetheless, in recent years several randomized clinical trials have shown that minimally-invasive surgery for colon cancer is a safe technique and that outcomes are equivalent to those obtained with open surgery.[4-6] Moreover, randomized trials comparing the short-term parameters of LC vs. open colectomy (OC) for colorectal cancer suggest advantages for the minimally invasive procedure over the conventional therapy. Most of these studies report that LC patients recover faster than OC patients, because of less pain, better pulmonary function, shorter duration of postoperative ileus, less fatigue and a better quality of life.[7-9]

Recently, some clinical studies found colon cancer LC more effective than OC in terms of morbidity and hospital stay[10] and similar disease free survival 3 years after surgery.[11] Moreover, an 11-year retrospective review with 5-years survival rates reports that overall survival and disease-free survival after LC for metastatic colon cancer are equivalent to and perhaps even better than the same parameters evaluated in patients treated with OC.[12] Kuhry, et al.[13] collected the data of 33 different randomized clinical trials comparing LC vs. OC in colorectal cancer. They found similar results about recurrence rate at the site of the primary tumor, the development of distant metastasis, cancer-related and overall mortality.

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As angiogenesis plays a crucial role in tissue repair, it is well known that a surgical procedure may enhance the local recruitment of some angiogenic cells and soluble growth factors[14,15] thus possibly influencing surgery outcome and cancer recurrence.

Different populations of endothelial cells play an important role in tumor angiogenesis and growth.[16] Circulating endothelial cell (CEC) kinetic and viability might correlate with clinical outcome in patients with cancer[16-20] CECs and their derived macroparticles (MP) are markers of vascular turnover and damage, circulating endothelial progenitors (CEPs) might play a role in vasculogenesis during cancer growth and recurrence after surgery.[16,21,22] We investigated the kinetic and viability of CECs, MPs, CEPs and some endothelial-specific and progenitor-associated RNA transcripts[23] in colon cancer patients undergoing LC or OC. These candidate biomarkers were previously found to be of clinical predictive/dynamic potential in a number of trials involving the administration of anti-angiogenic drugs to cancer patients.[18,19,22-24]

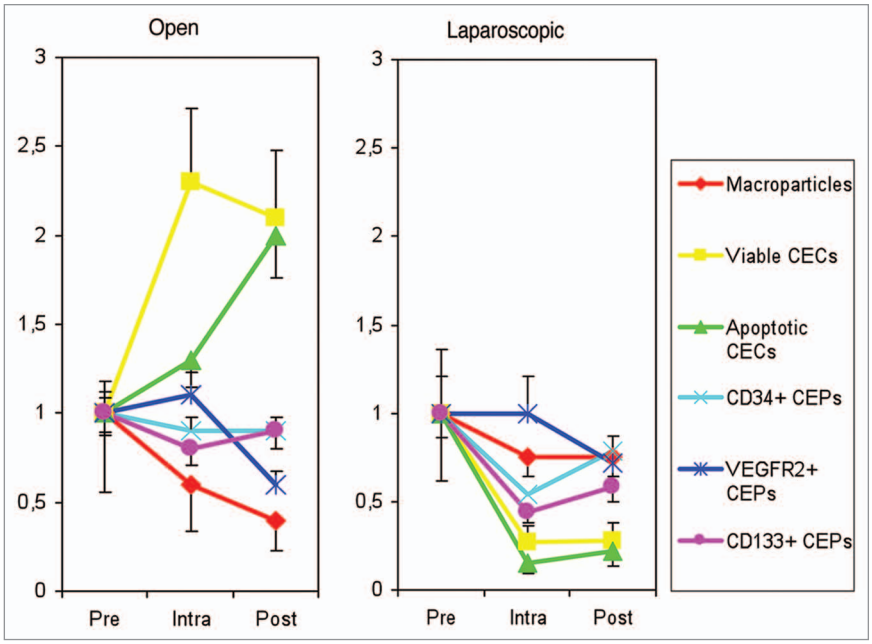

Figure 1. CEC, CEP and MP kinetics during and after OC and LC. Results are expressed as mean ± 1SD.

Results

Differences were found in the kinetics of angiogenic cells in OC vs. LC patients (**Fig. 1**). Viable, mature CECs (most likely reflecting vascular turnover) were significantly increased during and the day after OC ($p < 0.01$). Apoptotic CECs, most likely derived from damaged vessels, were significantly increased the day after OC ($p < 0.01$). Interestingly, both viable and apoptotic CECs were slightly decreased after LC ($p < 0.05$). CEP numbers, enumerated as $DNA^+/CD45dim/CD34^+$, $DNA^+/CD45dim/CD34^+/VEGFR2^+$ or $DNA^+/CD45dim/CD34^+/CD133^+$ cells, were not significantly increased or decreased by OC or LC. A non significant trend towards lower values of MP was observed after OC.

Among the angiogenic mRNA transcripts investigated, a significant decrease of circulating mRNA coding for VEGF-C, VEGF-D, VEGFR3 and PDGFR-beta was found after LC ($p < 0.01$, **Fig. 2**). After OC, we observed only a decrease of circulating RNA coding for VEGFR3 of borderline significance ($p = 0.044$). No statistically significant variations were observed in the circulating levels of other RNA transcripts studied after LC or OC.

Discussion

Following our previous observation that extended surgery can significantly increase peri-operative levels of angiogenic cytokines and growth factors when compared to more limited surgery,[24] the present study was designed to investigate differences related to the extent of surgery (OC vs. LC) in a variety of angiogenic cells, MP and angiogenesis-related RNA transcripts. Also, a recent report indicated that extensive surgery such as liver transplantation or resection is associated with the mobilization of hematopoietic and endothelial progenitors from the bone marrow to the peripheral blood.[25]

These results were also confirmed by Langenberg, et al.[26] who observed an instantaneous release of EPCs in response to increased levels of G-CSF, during and 24 hours after liver surgery.

Our results should be evaluated considering that in cancer patients the tumor itself is a major source of a variety of angiogenic cells and transcripts.[16,22] Thus, the kinetics of pre and post operative data involve the angiogenic profile before surgery, the removal of the tumor lesion containing angiogenic cells and transcripts and the wound healing process triggered by surgery. By means of a clinically validated procedure for CEC, CEP and MP enumeration by flow cytometry,[21,22] we observed that OC, but not LC, was associated with a significant post-operative increase in some angiogenic cells, namely CECs, either apoptotic (most likely the result of surgery-related vascular damage) and viable (most likely representing vascular remodelling).[16,22] The slight reduction observed in some angiogenic cells after LS was likely due to the removal of the highly vascularized tumor tissue. This reduction was not observed in OC patients most likely because of a more prominent wave of angiogenic cell mobilization which overcame the reduction due to the removal of the tumor lesion.

Regarding the kinetics of pre and post operative angiogenesis-related transcripts, circulating RNA coding for angiogenic genes VEGF-C, VEGF-D, VEGFR3 and PDGFR-B were significantly decreased after LC, likely because of the removal of the tumor lesion.

The diminished plasmatic level of the VEGF family is particularly interesting as it has been recently reported that during the

first 3 days after minimally invasive colorectal resection, the concentration of total soluble VEGF receptors decreases, rendering the plasma proangiogenic, as their binding proteins are available to bind more ECs.[27] Again, this decrease was not observed after OC, were a more pronounced wave of angiogenesis related to wound healing was likely. The trend towards higher levels of VEGF-D RNA after OC, albeit not statistically significant, was also likely related to wound healing.

Our data underline a previously unrecognized possible advantage of LC over OC, namely the downmodulation of different angiogenic cells and RNA transcripts after LC but not OC for colorectal cancer.

Considering also the emerging role of anti-angiogenic therapies for this type of cancer in medical oncology,[22,28] further studies are now warranted to better investigate the impact of surgery-related angiogenesis over colorectal cancer recurrence and patients' clinical outcome.

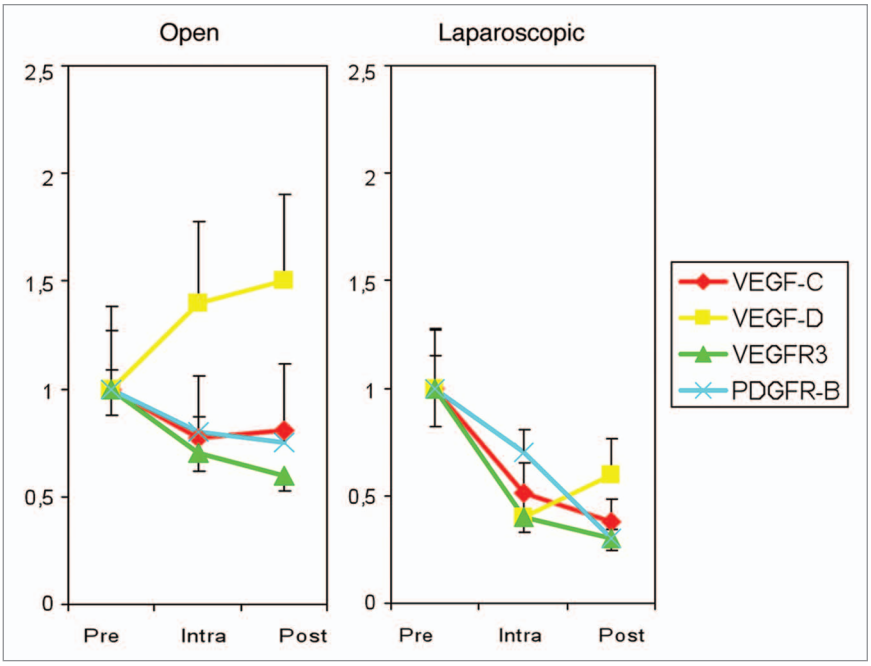

Figure 2. Kinetics of angiogenic RNA transcripts during and after OC and LC. For the sake of clarity, the figure shows only the parameters that significantly differed from baseline in at least one of the two arms of the study. Results are expressed as mean ± 1SD.

Patients and Methods

Patients. The trial was conducted at the European Institute of Oncology, Milan, Italy.

Patients (n = 24, see **Table 1** for details) had histologically proven colon-cancer. Patients were assigned to one arm or another based solely on the availability of a surgeon expert with minimally-invasive technique. We excluded emergency cases, previous colonic resection and patients who had prolonged pneumoperitoneum.

EDTA-containing blood was collected the day before surgery, intraoperatively and 24 hours after surgery. The trial was approved by the local Ethic Committee and the Institutional Review Board. Written informed consent was obtained.

Flow cytometry. CECs, CEPs and MP were measured by six color flow cytometry as previously described.[21] CECs were enumerated as $DNA^+/CD45^-/CD31^+/CD146^+$ cells. MP were enumerated as DNAlow/$CD45^-/CD31^+/CD146^+$ events. CEPs were defined as $DNA^+/CD45dim/CD34^+$ cells, $DNA^+/CD45dim/CD34^+/VEGFR2^+$ cells or $DNA^+/CD45dim/CD34^+/CD133^+$ cells.[16,22] The combination of Syto16 and 7-AAD was used to discriminate between viable (syto16bright/$7\text{-}AAD^-$) and apoptotic/necrotic (syto16weakly pos/$7\text{-}AAD^+$) cells. Results were evaluated normalizing the individual values to the baseline count.

Molecular biology. The number of mRNA copies coding for endothelial genes VE-cadherin, VEGFR-3, VEGFR-2, VEGF-A, VEGF-B, VEGF-C, VEGF-D and for angiogenesis-related genes PDGFR-β, and CD133 were enumerated by RT-PCR as described by Rabascio, et al.[23] Briefly, blood samples were lysed by NH_4Cl to remove red cells. Total RNA was extracted by the QIAamp RNA blood extraction kit (Qiagen, Chatsworth, CA). The Dnase-treated RNA (100 ng) was then converted into cDNA by High capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). RT-PCR amplification and detection were performed with the ABI Prism 7000 Sequence Detection Systems (Applied BioSystems).

The values were normalized on the average of the values of ten age-matched healthy volunteers.

Statistical analysis. Statistical comparisons were performed using the t-test, analysis of variance (ANOVA) and linear regression when data were normally distributed and the non-parametric analyses of Spearman and Mann-Whitney when data were not normally distributed. Values of p lower than 0.05 were considered as statistically significant.

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Table 1. Patients' characteristics

Treatment group	n	Age	Gender F/M	Tumor size	Lymphnode involvement	Tumor grade
Laparoscopic	13	69 ± 8	4/9	T0 = 1	N0 = 10	G1 = 1
				T1 = 1	N1 = 3	G2 = 4
				T2 = 5		G3 = 8
				T3 = 6		
Open	11	64 ± 8	2/9	T0 = 2	N0 = 7	G1 = 1
				T1 = 0	N1 = 2	G2 = 8
				T2 = 2	N2 = 2	G3 = 2
				T3 = 6		
				T4 = 1		
All	24	67 ± 8	6/18	T0 = 3	N0 = 17	G1 = 2
				T1 = 1	N1 = 5	G2 = 12
				T2 = 7	N2 = 2	G3 = 10
				T3 = 12		
				T4 = 1		

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