Effect of standard chemotherapy and antiangiogenic therapy on plasma markers and endothelial cells in colorectal cancer

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Introduction: The importance of the endothelium in angiogenesis and cancer is undisputed, and its integrity may be assessed by laboratory markers such as circulating endothelial cells (CECs), endothelial progenitor cells (EPCs), plasma von Willebrand factor (vWF), soluble E selectin, vascular endothelial growth factor (VEGF) and angiogenin. Antiantigenic therapy may be added to standard cytotoxic chemotherapy as a new treatment modality. We hypothesised that additional antiangiogenic therapy acts in a contrasting manner to that of standard chemotherapy on the laboratory markers.

Methods: We recruited 68 patients with CRC, of whom 16 were treated with surgery alone, 32 were treated with surgery followed by standard chemotherapy (5-flurouracil), and 20 were treated with surgery followed by standard chemotherapy plus anti-VEGF therapy (Avastin). Peripheral blood was taken before surgery, and again 3 months and 6 months later. CD34+/CD45-/CD146+/CECs and CD34+/CD45-/CD309[KDR]+ EPCs were measured by flow cytometry, plasma markers by ELISA.

Results: In each of the three groups, CECs and EPCs fell at 3 months but were back at pre-surgery levels at 6 months (P<0.05). VEGF was lower in both 3-and 6-month samples in the surgery-only and surgery plus standard chemotherapy groups (P<0.05), but in those on surgery followed by standard chemotherapy plus anti-VEGF therapy, low levels at 3 months (P<0.01) increased to pre-surgery levels at 6 months. In those having surgery and standard chemotherapy, soluble E selectin was lower, whereas angiogenin was higher at 6 months than at baseline (both P<0.05).

Conclusions: We found disturbances in endotheliod cells regardless of treatment, whereas VEGF returned to levels before surgery in those on antiangiogenic therapy. These observations may have clinical and pathophysiological implications.

Cancer and other solid tumors are the second most frequent cause of death in North America and Western Europe. In the UK, colorectal cancer is responsible for 14,000 of the 142,000 cancer deaths annually, with 38,000 new cases per year (Cancer Research UK, 2014; UK Government statistics, 2014). Tumor angiogenesis is implicit in the functions of the endothelium, which not only contributes to the structure of the vessel wall but also promotes further neovascularisation (in the local cancer milieu) by producing angiogenic mediators of its own, such as vascular endothelial growth factor (VEGF) and angiogenin (Folkman 1990; Takahashi et al., 1995; Shimoyama et al., 1999). VEGF is a target of therapy for colorectal and many other forms of cancer (Koutras et al, 2011), whereas raised angiogenin in colorectal cancer is associated with disease stage and outcome after surgery (Shimoyama et al., 1999).

Furthermore, there are several other lines of evidence of endothelial activity or perturbation in cancer, such as increased release of endothelial cell-specific molecules von Willebrand factor (vWF) and soluble E selectin into the plasma, as are present in colorectal cancer (Gil-Bazo et al, 2005; Sata et al., 2010).

More recently, increased numbers of endotheloid cells (cells with endothelial-like morphology and phenotype), have been described in peripheral blood of patients with various solid
tumours including colorectal cancer, the origins of which are the subject of much debate (Beerepoot et al, 2004; Fürstenberger et al, 2006; Rowand et al, 2007; Dome et al, 2008). These cells may arise from existing normal and/or tumour vasculature (known as mature circulating endothelial cells (CECs)) or may represent endothelial progenitor cells (EPCs) derived from stem cells (probably from the bone marrow) that may contribute to neovascularisation (Blann and Pretorius, 2006). Differences in EPCs and CECs numbers may therefore represent the balance of injury and repair to blood vessels, and/or the neovascularisation required of tumour angiogenesis. If endothelial cells are indeed crucial to neovascularisation, this would make them a legitimate target (Ding et al, 2008; Mancuso and Bertolini, 2010). However, we have argued that one or even two markers of vascular perturbation used in isolation are insufficient to fully describe changes to the endothelium and in angiogenesis in cancer, and that a combination of types of methods (e.g. cell markers should be used alongside plasma markers) will provide a more complete view of tumour biology (Blann et al, 2011).

Standard treatment of cytotoxic chemotherapy alone has been challenged by the concurrent use of antiangiogenic therapy (Hurwitz et al, 2004; Gil-Bazo et al, 2005; Fürstenberger et al, 2006; Kourtas et al, 2011). We hypothesised that adding antiangiogenic therapy produces different responses in cellular (EPCs, CECs) and plasma (vWF, soluble E selectin) markers of vascular perturbation, and plasma growth factors (VEGF, angiogenen). We tested our hypothesis in three groups of patients with colorectal cancer undergoing treatment (surgery alone, surgery plus chemotherapy alone, surgery plus chemotherapy and antiangiogenic therapy), taking blood before surgery and again 3 and 6 months later.

MATERIALS AND METHODS

Patients were recruited from among those scheduled to undergo surgery for colorectal cancer at a large urban University Hospital. Standard surgical primary tumour resections were carried out with associated mesocolic/mesorectal lymphadenectomy. Specimens were sent for routine histology and the Dukes’ stage derived according to standard criteria (Dukes, 1932; Astler and Coller, 1954). Exclusion criteria were concurrent inflammatory connective tissue disease (e.g. rheumatoid arthritis), other neoplasia, marked haematological (full blood count) or biochemical (U&Es) abnormalities, for example, anaemia and renal disease (eGFR <30) and certain medications (antibiotics, NSAIDs, HRT and anticoagulant therapy). Local research ethics committee approval and informed written consent from all subjects were obtained.

Three months after surgery, a second blood sample was obtained. A clinical decision was then taken to proceed with no further treatment, with local standard chemotherapy based on 5-fluorouracil with or without antiangiogenic therapy. Chemotherapy was based on a modified de Gromant regime (leucovorin [folinic acid] + 5-fluorouracil [5-FU]) regime. For stage 2 (Dukes’ B) disease this was 5-FU 1200 mg m⁻² IV bolus, followed by 5-FU 1200 mg m⁻² per day IV x 2 days (total 2400 mg m⁻²) as a 46–48 h continuous infusion, repeated every 2 weeks. Oxaliplatin [Eloxatin] + leucovorin [folinic acid] + 5-fluorouracil [5-FU]) was mainly for stage 3 (Dukes’ C) disease as follows. Day 1: Oxaliplatin 85 mg m⁻² IV over 2 h + leucovorin 400 mg m⁻² IV over 2 h, followed by 5-FU 400 mg m⁻² IV bolus, followed by 5-FU 1200 mg m⁻² per day IV x 2 days (total 2400 mg m⁻²) as a 46–48 h continuous infusion, repeated every 2 weeks (Cheeseman et al, 2002, 2008; André et al, 2004, 2009; Twelves et al, 2005). In some cases, treatment was days 1–14 Capecitabine [Xeloda] 1250 mg m⁻² orally twice daily for 2 weeks. The cycle was repeated every 3 weeks for 8 cycles over a 6 months altogether.

For those on antiangiogenic therapy, bevacizumab (Hurwitz et al, 2004) (5 mg kg⁻¹) was given IV on the first day of each 3 week cycle, and then 5 mg kg⁻¹ every 2 weeks. A third blood sample was obtained from subjects in all three group after a further 3 months.

Blood was taken for CECs and EPCs, and for plasma markers. For the latter, blood was centrifuged at 2500 rpm (=1000 g) for 20 min to provide plasma for VEGF, angiogenen, soluble E selectin (CD69E) and vWF. Plasma was stored frozen at −70 °C for batch analysis by ELISA (R&D Systems, Abingdon, UK). Dako-Patts, Ely, Cambs, UK). Intra-assay and inter-assay coefficients of variation (CVs) were <5% and <10%, respectively.

EPCs and CECs were analysed by flow cytometry (FACScalibur, Becton Dickinson, Oxford, UK) (Goon et al, 2009; Blann et al, 2011). In brief, to 200 μl of blood was added 10 μl each of fluorochrome-labelled antibodies to CD45, CD146, CD34 and CD309 (all Becton Dickinson except anti-CD309, R&D Systems). Blood was incubated in the dark at room temperature for 20 min, then 3-ml red cell lysing/fixative solution (Becton Dickinson) was added and the tube and incubated for 15 min on the bench. Excess reagents were washed out with cycles of centrifugation (200 g for 5 min) and 3 ml of PBS solution. After the final wash, 0.5 ml of PBS was added and the suspension was applied to the FACS calibur.

The CellQuest Pro software of Apple G4 computer was used to determine cell counts with a minimum of 100,000 events. White blood cells were identified and excluded by SSC and FSC in conjunction with CD45-PerCP as it is established that some white blood cells may also express endothelial and/or stem cell markers (Beerepoot et al, 2004; Fürstenberger et al, 2006; Rowand et al, 2007; Ding et al, 2008; Dome et al, 2008; Mancuso and Bertolini, 2010). CD34-APC +ve cells were then gated and subsequently re-probed in separate analyses with CD309-PE and CD45-PerCP for EPCs or with CD146-FITC and CD45-PerCP for CECs. Intra- and inter-assay CVs were <24% for EPCs <47% for CECs, <40% for EPCs and <60% for CECs, respectively.

Statistics. Data are presented as mean and s.d. (when normally distributed) or median and interquartile range (non-normally distributed). Comparison of the three groups at baseline was by analysis of variance (ANOVA) or the Kruskal–Wallis test. The χ² test was used for categorical data. Differences over the two time points were sought using paired t-test or Wilcoxon’s test, at three time points by a general linear model ANOVA. We included the routine white blood cell count, known to be raised in colorectal cancer (Kemeny and Braun, 1983), in our analyses to provide a perspective for CEC and EPC results. A sample size of 66 yields 2P<0.02 and 1– beta = 0.85 to robustly defend a (Spearman) correlation coefficient of r>0.4. All analyses were performed on Minitab release 16 and P<0.05 was taken to assume significance.

RESULTS

Clinical and demographic details of the patients are shown in Table 1. There were no differences in sex, age, blood pressure, heart rate, family history of cancer, smoking and body mass index between the groups. There was, however, a difference in distribution of Dukes’ stage between the groups. Subjects with a higher Dukes’ stage were more likely to have chemotherapy with or without antiangiogenic therapy. Table 2 shows research data before surgery from each of the three treatment groups. With the exception of soluble E selectin being higher in those on standard chemotherapy. There were no differences in these indices at baseline between the groups. Table 3 shows changes in research indices in the entire cohort of 68 patients before and 3 months after their surgery but before additional adjunct therapy. All indices except total CD34 +ve cells were lower after surgery.
Table 1. Clinical and demographic details of the subjects

|                      | Group 1 surgery only (n = 16) | Group 2 standard chemotherapy (n = 32) | Group 3 anti-VEGF therapy (n = 20) | P-value |
|----------------------|-------------------------------|---------------------------------------|-----------------------------------|---------|
| Age (years)          | 71.2 (9.3)                    | 70.9 (7.9)                            | 70.7 (7.9)                        | 0.988   |
| Sex male/female (%)  | 9/7                           | 18/14                                 | 10/10                             | 0.895   |
| BMI (kg m\(^{-2}\))  | 28.4 (3.1)                    | 27.7 (2.6)                            | 26.2 (2.4)                        | 0.041   |
| Systolic blood pressure (mm Hg) | 121 (13) | 123 (9) | 122 (10) | 0.876   |
| Diastolic blood pressure (mm Hg) | 74 (8) | 75 (10) | 74 (7) | 0.732   |
| Dukes’ stage (A/B/C/D) (n) | 9/5/1/1 | 2/13/8/9 | 0/5/11/4 | <0.001 |
| Major tumour site (colon/rectum) (n) | 12/4 | 22/10 | 10/10 | 0.239   |
| Tumour size (mm)     | 37 (17)                       | 47 (24)                               | 43 (17)                           | 0.282   |
| Number of nodes excised (n) | 10.5 (8.5–12) | 12 (8.25–18.7) | 11.5 (10.25–18.75) | 0.321   |

Data are mean (s.d.), median (interquartile range), absolute number (n) or percentage. Analysed by ANOVA, Kruskal–Wallis or \( \chi^2 \) as appropriate.

Table 2. Research indices at baseline

|                      | Group 1 surgery only (n = 16) | Group 2 standard chemotherapy (n = 32) | Group 3 surgery plus standard chemotherapy and anti-VEGF therapy (n = 20) | P-value |
|----------------------|-------------------------------|---------------------------------------|--------------------------------------------------------------------------|---------|
| Circulating endothelial cells (cells ml\(^{-1}\)) | 4 (0–19)                     | 12 (0–19)                             | 11 (7–48)                                                                | 0.746   |
| Endothelial progenitor cells (cells ml\(^{-1}\)) | 13 (0–39)                    | 16 (9–44)                             | 21 (10–39)                                                               | 0.749   |
| Von Willebrand factor (IU dl\(^{-1}\)) | 115 (22)                     | 123 (33)                              | 121 (30)                                                                 | 0.659   |
| Soluble E selectin (ng ml\(^{-1}\)) | 31 (9)                       | 34 (12)*                              | 26 (8)*                                                                  | 0.029   |
| VEGF (pg ml\(^{-1}\)) | 120 (10–494)                  | 320 (30–1200)                         | 500 (40–1000)                                                            | 0.160   |
| Angiogenin (ng ml\(^{-1}\)) | 308 (75)                     | 282 (97)                              | 319 (97)                                                                 | 0.231   |
| CD34 + ve cells (cells ml\(^{-1}\)) | 849 (638–1167)              | 781 (417–1315)                        | 781 (585–1123)                                                           | 0.839   |
| White cell count (x10\(^6\)ml\(^{-1}\)) | 6.7 (1.6)                    | 7.9 (2.2)                             | 6.7 (1.5)                                                                | 0.046*  |

Data are mean (s.d.) or median (interquartile range). Analysed by ANOVA, *P < 0.05 (Tukey’s post hoc test). Reference ranges from a control population (reference 34) are circulating endothelial cells 0 (0–8), endothelial progenitor cells 7 (0–12), von Willebrand factor 110 (31), soluble E selectin 22 (10), VEGF 32 (0–82), angiogenin 143 (07–175) and white cell count 5.8 (1.3). We do not have a reference range for CD34 + ve cells.

*p*No significant inter-group differences.

Table 3. Research indices before and 3 months after surgery in 68 patients

|                      | Before surgery | Three months after surgery | P-value |
|----------------------|----------------|---------------------------|---------|
| Circulating endothelial cells (cells ml\(^{-1}\)) | 10 (0–20) | 0 (0–10) | 0.001 |
| Endothelial progenitor cells (cells ml\(^{-1}\)) | 17 (2.3–40) | 0 (0–9) | <0.001 |
| Von Willebrand factor (IU dl\(^{-1}\)) | 121 (30) | 110 (25) | 0.018 |
| Soluble E selectin (ng ml\(^{-1}\)) | 31 (11) | 26 (12) | 0.024 |
| VEGF (pg ml\(^{-1}\)) | 310 (30–771) | 23 (12.5–51) | <0.001 |
| Angiogenin (ng ml\(^{-1}\)) | 301 (94) | 238 (100) | <0.001 |
| CD34 + ve cells (cells ml\(^{-1}\)) | 74 (51.5–109.5) | 96.5 (58–131) | 0.065 |
| White cell count (x10\(^6\)ml\(^{-1}\)) | 7.3 (2.0) | 5.4 (1.5) | <0.001 |

Data are mean (s.d.) or median (interquartile range). Analysed by paired t-test or Wilcoxon’s test.

Table 4 shows data from 16 patients treated with surgery alone. Although overall, CEC numbers changed significantly, this was so minor that there was no specific inter-group difference. EPCs and VEGF fell from baseline to 3 months (both \( P < 0.05 \)). At 6 months, VEGF remained low (\( P < 0.05 \)), but the EPC count was not different from baseline. There were no significant changes in vWf, soluble E selectin, angiogenin, CD34 + ve cells or the white cell count. Changes in EPC and CEC counts are shown in Figures 1A and 2A, respectively.

Table 5 shows data from 32 patients treated with standard chemotherapy. CECs were lower at 3 months (\( P < 0.05 \)), but at 6 months, were no different to baseline levels. EPC numbers fell at 3 months (\( P < 0.01 \)) but had returned to baseline levels at 6 months (\( P < 0.05 \) to numbers at 3 months). Soluble E selectin was lower at 6 months compared with baseline and 3 months (both \( P < 0.05 \)). VEGF and the white cell count were lower at 3 and 6 months compared with baseline (both \( P < 0.01 \)). Levels of angiogenin fell from baseline to 3 months (\( P < 0.05 \)), but then increased from 3 months to 6 months (\( P < 0.01 \)). Changes in EPC and CEC counts are shown in Figures 1B and 2B, respectively.

Table 6 shows data from patients on standard chemotherapy and antiangiogenic therapy. The numbers of CECs fell to 3 months (\( P < 0.01 \)), then increased to 6 months (\( P < 0.05 \)). The numbers of EPC and VEGF levels fell to 3 months and then increased to 6 months (all \( P < 0.01 \)). There were no changes in vWf or...
angiogenin. CD34+ cell numbers were lower at 6 months than at baseline, whereas the white blood cell count fell at 3 months (P<0.01) and at 6 months (P<0.02) compared with baseline. Changes in EPC and CEC counts are shown in Figures 1C and 2C, respectively.

At baseline, there were significant Spearman correlations between EPCs and CECs (r = 0.7, P < 0.001), between VEGF and CECs (r = 0.55, P < 0.001) and between VEGF and EPCs (r = 0.69, P < 0.001). At 3 months, these relationships were still present but were less strong at r = 0.52 (P < 0.001), r = 0.28 (P = 0.019) and r = 0.25 (P = 0.037), respectively, although the latter two may be false negatives as we are only powered for P<0.05, baseline to 6-month point.

DISCUSSION

Angiogenesis, possibly driven by growth factors such as VEGF and angiogenin (Folkman, 1990; Takahashi et al, 1995; Shimoyama et al, 1999) is a leading factor in the pathophysiology of cancer. Increased number of endothelial cells in the blood may also have a role in this disease (Beerepoot et al, 2004; Fürstenberger et al, 2006; Rowand et al, 2007; Dome et al, 2008). EPCs may be surrogates of the effects of anti-VEGF therapy (Takahashi et al, 1995; Gupta, 2007; Ronzoni et al, 2010; Matusaka et al, 2011), as VEGF may partially drive EPCs mobilisation from the bone marrow (Asahara et al, 1999; Li et al, 2006). Indeed, the strong relationships of VEGF with CECs and EPCs at baseline to some extent support this hypothesis, although the weakening of these relationships as treatment proceeds casts some doubt. As research in this area is hindered by the lack of consensus as to which CD markers best define these cells (Takahashi et al, 1995; Asahara et al, 1999; Beerepoot et al, 2004; Blann and Pretorius, 2006; Fürstenberger et al, 2006; Li et al, 2006; Gupta, 2007; Rowand et al, 2007; Ding et al, 2008; Dome et al, 2008; Gooen et al, 2009; Mancuso and Bertolini, 2010; Bellows et al, 2011; Blann et al, 2011; Ramcharan et al, 2013), cross-comparisons can be difficult. Nevertheless, endothelial cells measured before treatment seem to be good markers of outcome (Ronzoni et al, 2010; Matusaka et al, 2011) and reflect Dukes’ stage (Ramcharan et al, 2013). However, few studies have examined a comprehensive panel of vascular and growth factor markers before and after standard chemotherapy and antiangiogenic therapy.

In the entire group, reductions in all research indices (except CD34+ cells) 3 months after surgery were all as expected, and, despite reduced power, this reduction was found in all three subgroups before additional therapy. These findings fit with the general hypothesis of a link between these markers and the tumour load. However, a consistent finding was that the numbers of both

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**Table 4. Research indices in 16 patients in group 1 (surgery only)**

|                      | Baseline       | 3 months       | 6 months       | P-value |
|----------------------|----------------|----------------|----------------|---------|
| Circulating endothelial cells (cells ml⁻¹) | 4 (0–19)       | 0 (0–9)        | 8 (0–10)       | 0.045   |
| Endothelial progenitor cells (cells ml⁻¹)  | 13 (0–39)      | 0 (0–9)        | 8 (0–10)       | 0.036   |
| Von Willebrand factor (IU dl⁻¹)            | 115 (22)       | 106 (19)       | 118 (30)       | 0.441   |
| Soluble E selectin (ng ml⁻¹)               | 31 (9)         | 26 (9)         | 29 (11)        | 0.302   |
| VEGF (pg ml⁻¹)                            | 120 (10–494)   | 23 (6–23)      | 16 (5–82)      | 0.024   |
| Angiogenin (ng ml⁻¹)                       | 308 (75)       | 235 (97)       | 300 (96)       | 0.061   |
| CD34+ve cells (cells ml⁻¹)                 | 849 (638–1167) | 1083 (679–1603)| 918 (500–1269)| 0.395   |
| White cell count (x10⁶ ml⁻¹)               | 6.7 (1.6)      | 5.7 (1.7)      | 6.2 (1.3)      | 0.175   |

Data are mean (s.d.) or median (interquartile range). P-value by ANOVA.

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**Table 5. Research indices in 32 patients in Group 2 (surgery followed by standard chemotherapy)**

|                      | Baseline       | 3 months       | 6 months       | P-value |
|----------------------|----------------|----------------|----------------|---------|
| Circulating endothelial cells (cells ml⁻¹) | 12 (0–19)      | 3 (0–11)       | 10 (0–12)      | 0.019   |
| Endothelial progenitor cells (cells ml⁻¹)  | 16 (9–44)      | 0 (0–11)       | 10 (8–15)      | <0.001  |
| Von Willebrand factor (IU dl⁻¹)            | 123 (33)       | 106 (25)       | 119 (28)       | 0.08    |
| Soluble E selectin (ng ml⁻¹)               | 34 (12)        | 38 (9)         | 24 (10)        | 0.007   |
| VEGF (pg ml⁻¹)                            | 320 (30–1200)  | 30 (20–287)    | 30 (21–310)    | 0.001   |
| Angiogenin (ng ml⁻¹)                       | 282 (97)       | 231 (93)       | 346 (109)      | <0.001  |
| CD34+ve cells (cells ml⁻¹)                 | 781 (417–1315) | 839 (583–1212)| 686 (361–1056)| 0.304   |
| White cell count (x10⁶ ml⁻¹)               | 7.9 (2.2)      | 5.4 (1.3)      | 5.8 (2.0)      | <0.001  |

Data are mean (s.d.) or median (interquartile range). P-value by ANOVA/general linear model/Tukey’s post hoc test.

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www.bjcancer.com | DOI:10.1038/bjc.2014.491
In patients undergoing surgery alone

In patients undergoing surgery plus chemotherapy

In patients undergoing surgery plus chemotherapy and anti-VEGF therapy

*Overall P-value. aBetween baseline and 3 months; bBetween 3 and 6 months; cBetween baseline and 6 months. Data median (interquartile range).

Figure 1. Changes in EPC numbers. (A) In patients undergoing surgery alone. (B) In patients undergoing surgery plus chemotherapy. (C) In patients undergoing surgery plus chemotherapy and anti-VEGF therapy.

Figure 2. Changes in CEC numbers. (A) In patients undergoing surgery alone. (B) In patients undergoing surgery plus chemotherapy. (C) In patients undergoing surgery plus chemotherapy and anti-VEGF therapy.

*Overall P-value. aBetween baseline and 3 months; bBetween baseline and 6 months. Data median (interquartile range).
endothelial cells increased regardless of the presence or absence of adjunct chemotherapy and antiangiogenic therapy. We are unable to offer a simple explanation for the increase in both cell populations after an additional 3 months. It may be that the rise in both EPCs and CECs in those not going on chemotherapy reflects the response of the body to some residual unexposed tumour and/or the physiology of repair. The rise in EPCs and CECs in those on both regimes of pharmacotherapy may be a drug-related response to these agents, or it may reflect the failure of these drugs to suppress the increase in cell numbers after surgery. However, Willett et al (2005) reported that CECs numbers fell in five rectal cancer patients after 3 days of 5 mg kg$^{-1}$ drug-related response to these agents, or it may reflect the failure of the entire cohort, this was due to a marked fall in those on surgery plus standard chemotherapy alone (the largest group of 32 patients): there was no significant change in those on surgery alone ($n = 16$) or those on surgery plus standard chemotherapy plus anti-VEGF therapy ($n = 20$). Once more, small number of patients per group leads us to be cautious in speculating that we are witnessing a genuine reduction in angiogenesis per se, in only one of the three treatment groups, or simply a nonspecific response of the endothelium to the different types of treatment.

We accept the limitation of the small number of patients in each group, and despite this, some of the changes were highly significant. We therefore stand by what we feel is our major result, which is that numbers of CECs and EPCs fall markedly 3 months after surgery, but increase after a further 3 months regardless of treatment. This calls into doubt the value of a single measurement of these cells as markers of disease activity or outcome in either the short or long term.

### REFERENCES

André T, Boni C, Mounedji-Boudiaf L, Navarro M, Tabernero J, Hickish T, Topham C, Zaninelli M, Clingan P, Bridgewater J, Tabah-Fisch I, de Gramont A (2004) Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. *N Engl J Med* **350**: 2343–2351.

André T, Boni C, Navarro M, Tabernero J, Hickish T, Topham C, Bonetti A, Clingan P, Bridgewater J, Rivera F, de Gramont A (2009) Improved overall survival with oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment in stage II or III colon cancer in the MOSAIKC trial. *J Clin Oncol* **27**: 3109–3116.

Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, Inai Y, Silver M, Isner JM (1999) VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J* **18**: 3964–3972.

Asteller VB, Collier FA (1954) The prognostic significance of direct extension of carcinoma of the colon and rectum. *Ann Surg* **139**: 846.

Table 6. Research indices in 20 patients in Group 3 (surgery followed by standard chemotherapy and antiangiogenic therapy)

|                     | Baseline | 3 months | 6 months | P-value |
|---------------------|----------|----------|----------|---------|
| Circulating endothelial cells (cells ml$^{-1}$) | 21 (7–60) | 0 (0–13) | 11 (7–48) | 0.005$^a$ |
| Endothelial progenitor cells (cells ml$^{-1}$) | 21 (10–39) | 0 (0–0) | 12 (7–32) | <0.01$^b$ |
| Von Willebrand factor (IU dl$^{-1}$) | 121 (30) | 122 (26) | 122 (15) | 0.943 |
| Soluble E selectin (ng ml$^{-1}$) | 26 (8) | 22 (15) | 29 (12) | 0.195 |
| VEGF (mg ml$^{-1}$) | 500 (40–1000) | 30 (23–55) | 770 (630–1900) | <0.01$^b$ |
| Angiogenin (ng ml$^{-1}$) | 319 (97) | 255 (117) | 317 (87) | 0.074 |
| CD34 + ve cells (cells ml$^{-1}$) | 781 (585–1123) | 595 (427–1139) | 291 (192–535) | 0.003$^a$ |
| White cell count (x10$^9$ ml$^{-1}$) | 6.7 (1.5) | 5.1 (1.7) | 5.3 (1.4) | 0.003$^d$ |

Data are mean (s.d.) or median (interquartile range).

$^a$P<0.01, baseline to 3 months, P<0.05, 3 months to 6 months.

$^b$P<0.01, baseline to 6 months.

$^c$P<0.01, baseline to 3 months.

$^d$P<0.01, baseline to 6 months.
Willett CG, Boucher Y, Duda DG, di Tomaso E, Munn LL, Tong RT, Kozin SV, Petit L, Jain RK, Chung DC, Sahani DV, Kalva SP, Cohen KS, Scadden DT, Fischman AJ, Clark JW, Ryan DP, Zhu AX, Blaszkowsky LS, Shellito PC, Mino-Kenudson M, Lauwers GY (2005) Surrogate markers for antiangiogenic therapy and dose-limiting toxicities for bevacizumab with radiation and chemotherapy: continued experience of a phase I trial in rectal cancer patients. J Clin Oncol 23: 8136–8139.

Werther K, Christensen IJ, Brunner N, Nielsen HJ (2000) Soluble vascular endothelial growth factor levels in patients with primary colorectal carcinoma. The Danish RANX05 Colorectal Cancer Study Group. Eur J Surg Oncol 26: 657–662.

Yang JC, Haworth L, Sherry RM, Hwu P, Schwartzentruber DJ, Topalian SL, Steinberg SM, Chen HX, Rosenberg SA (2003) A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. New Engl J Med 349: 427–434.

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