The relationships between cellular components of the peritumoural inflammatory response, clinicopathological characteristics and survival in patients with primary operable colorectal cancer

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BACKGROUND: The host inflammatory response is an important determinant of cancer outcome. We examined different methods of assessing the local inflammatory response in colorectal tumours and explored relationships with both clinicopathological characteristics and survival.

METHODS: Cohort study of patients (n = 130) with primary operable colorectal cancer and mature follow-up. Local inflammatory response at the invasive margin was assessed with: (1) a semi-quantitative assessment of peritumoural inflammation using Klintrup–Makinen (K–M) grading and (2) an assessment of individual immune cell infiltration (lymphocytes, plasma cells, neutrophils, macrophages and eosinophils).

RESULTS: The peritumoural inflammatory response was K–M low grade in 48% and high grade in 52%. Inflammatory cells were primarily macrophages, lymphocytes and neutrophils with relatively few plasma cells or eosinophils. On univariate analysis, K–M grade, lymphocyte infiltration and plasma cell infiltration were associated with cancer-specific survival. On multivariate analysis, only systemic inflammatory response, TNM (tumour, node and metastases) stage, venous invasion, tumour necrosis and K–M grade were independently associated with cancer-specific survival. There was no relationship between local infiltration of inflammatory cells and a systemic inflammatory response. However, high K–M grade, lymphocyte infiltration and plasma cell infiltration were associated with a number of favourable pathological characteristics, including an absence of venous invasion.

CONCLUSION: Infiltration of inflammatory cells in the invasive margin of colorectal tumours is beneficial to survival. The adaptive immune response appears to have a prominent role in the prevention of tumour progression in patients with colorectal cancer.

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Colorectal cancer is the second most common cause of cancer death in western Europe and North America. It is now recognised that the long-term survival of patients with colorectal cancer is dependent not only on pathological stage but also on complex interactions between tumour- and patient-related factors. In particular, the host inflammatory responses, both systemic and local, are important determinants of cancer outcome. A pronounced systemic inflammatory response has been consistently associated with reduced survival in a number of solid organ tumour types, including colorectal cancer, and has now been rationalised into a simple and reliable prognostic tool, termed the modified Glasgow Prognostic Score (mGPS; Ishizuka et al, 2007; Roxburgh and McMillan, 2010).

In contrast to the systemic response, local infiltration of inflammatory cells in the tumour microenvironment is associated with improved survival in patients with colorectal cancer. However, despite extensive investigation over a 40-year period, a reliable measure of the local inflammatory cell infiltrate has yet to be incorporated into clinical practice (Jass et al, 1987; Pages et al, 2005; Forssell et al, 2007). To establish routine clinical utility there is, therefore, a need to standardise the assessment of the local inflammatory cell response in colorectal tumours.

A logical starting point would be to compare the prognostic value and clinicopathological associations of individual immune cell types with a more generalised assessment of local inflammation. Indeed, a global assessment of peritumoural inflammatory cell infiltrate, using routine haematoxylin and eosin (H&E)-stained sections, has been proposed by Klintrup et al (2005) (Klintrup–Makinen grade, K–M grade) and independently validated by Roxburgh et al (2009).

The aim of the present study, therefore, was to examine the relationships between overall K–M grade, individual inflammatory
cell components and survival in patients with primary operable colorectal cancer.

MATERIALS AND METHODS

Patients with colorectal cancer who, on the basis of preoperative staging and laparotomy findings, were considered to have undergone potentially curative resection of colorectal cancer (stages I–III) between January 1997 and December 2006 in a single surgical unit at Glasgow Royal Infirmary were included. The patients were identified from a prospectively maintained database of elective and emergency resections. Exclusion criteria were: (i) clinical evidence of active infection, (ii) presence of a chronic inflammatory condition, (iii) preoperative chemotherapy and (iv) death within 30 days of surgery.

Routine laboratory measurements of haemoglobin, differential white blood cell count, albumin and C-reactive protein (CRP) were recorded before surgery. Using local reference ranges, anaemia was defined as a haemoglobin concentration <13.0 g dl\(^{-1}\) in men and <11.5 g dl\(^{-1}\) in women, and severe anaemia as <11.0 g dl\(^{-1}\) in men and <10.0 g dl\(^{-1}\) in women. The systemic inflammatory response was assessed using the mGPS and has been described previously (McMillan, 2008). Briefly, patients with both a raised CRP level (>10 mg l\(^{-1}\)) and a low albumin (<35 g l\(^{-1}\)) were allocated a score of ‘2’. Patients with a raised level of CRP alone were scored ‘1’. Patients with normal values or hypoalbuminaemia alone were scored ‘0’.

The peritumoural inflammatory cell infiltrate had also been assessed previously in the cohort according to the K–M criteria (inter-observer intraclass correlation coefficient 0.81; Roxburgh et al, 2009). Briefly, original H&E-stained sections (median 3, range 2–5) were selected from areas of the central tumour felt to represent the maximum depth of tumour invasion. The invasive margin of each tumour was then scored on a four-point scale. A score of ‘0’ indicated no increase in inflammatory cells; score ‘1’ denoted a mild or patchy increase, score ‘2’ a prominent inflammatory reaction, and score ‘3’ a florid ‘cup-like’ inflammatory infiltrate. The peritumoural inflammatory cell response was subsequently classified as low grade (score 0–1) or high grade (score 2–3).

The following method was then used to identify individual inflammatory cells. The original H&E-stained sections used for the K–M grading were retrieved from pathology archives and a representative section was chosen for a more detailed analysis. This section was converted to electronic format using a high-resolution digital scanner (Slidepath Digital Image Hub v3, Wetzlar, Germany) before five distinct areas (560 mm\(^2\) × 250 mm\(^2\)) were selected at intervals along the invasive margin. Gridlines were scored ‘1’. Patients with normal values or hypoalbuminaemia alone were scored ‘0’.

The tumours were staged according to the fifth edition of the tumour, node and metastases (TNM) classification (Fleming et al, 1997). Additional pathological data were taken from reports issued at the time of resection. With regard to venous invasion, cases in the present study that predated the introduction of routine elastica staining at Glasgow Royal Infirmary in 2003 were stained and reported retrospectively. Tumour necrosis had been assessed in the cohort previously according to published methodology (Richards et al, 2011). Briefly, the extent of necrosis in the central tumour was assessed semi-quantitatively and graded as ‘absent’ (none), ‘focal’ (<10% of tumour area), ‘moderate’ (10–30%) or ‘extensive’ (>30%).

The inflammatory cell types were divided into two equal groups termed ‘low’ and ‘high’ based on the median cell count per 0.018 mm\(^2\). Grouping of other variables was carried out using standard or previously published thresholds. Associations between categorical and continuous variables were examined using \(\chi^2\) tests for linear trend and non-parametric tests. Survival analyses were performed using the Kaplan–Meier method and Cox proportional hazards regression. Variables significant on univariate analysis were entered into a multivariate model using a backwards conditional method. \(P\)-value <0.050 was considered statistically significant. Statistical analyses were performed using SPSS version 19.0 (IBM SPSS, Chicago, IL, USA).

RESULTS

A total of 130 patients who underwent potentially curative resection of colorectal cancer were included. The majority of patients were 65 years or older (68%) with a similar number of males (52%) and females (48%). Most operations were elective (94%) and were carried out for both colon (68%) and rectal cancer (32%). The preoperative systemic inflammatory response was graded as mGPS 0 in 68 patients (52%), 1 in 47 (36%) and 2 in 15 (12%). Original pathological reports classified the 8% of the tumours as stage I, 49% as stage II and 43% as stage III. Using elastin staining, there was evidence of intra- or extramural venous invasion in 43 of the tumours (33%). In the postoperative period, a total of 38 patients (29%) received adjuvant chemotherapy.

Application of the K–M criteria graded the peritumoural inflammatory cell response as ‘low grade’ in 63 patients (48%) and ‘high grade’ in 67 patients (52%). The distribution of individual inflammatory cell types in the invasive margin are summarised in Table 1. The cells identified were primarily macrophages, lymphocytes and neutrophils with relatively few plasma cells or eosinophils (Table 1).

The relationships between overall K–M grade and individual inflammatory cell types are shown in Figure 1. There were significant relationships between high K–M grade and increased numbers of lymphocytes \((P<0.001)\), plasma cells \((P<0.001)\), neutrophils \((P<0.01)\) and eosinophils \((P<0.01)\). There was no relationship between K–M grade and macrophage count (Figure 1).

The median follow-up for the survivors was 105 months (range 55–163). During this period, 37 patients died from colorectal cancer and 34 patients died from other causes. The survival analyses for K–M grade and individual inflammatory cell types are shown in Table 2. On univariate analysis, K–M grade \((P<0.01)\), lymphocyte, plasma cell, neutrophil, macrophage (including mast cells), eosinophil or other (which included neoplastic, stromal, endothelial, necrosed or unidentified cells). The cells were counted using image analysis software (Image) available at http://rsweb.nih.gov/ij/). A total of 20 cases were scored independently by two observers to confirm consistency of scoring. The inter-observer intraclass correlation coefficients for each cell type were: lymphocytes 0.92, plasma cells 0.80, neutrophils 0.65, macrophages 0.40 and eosinophils 0.92.

Patients were followed up for 5 years after surgery. Information on date and cause of death was cross-checked with that received by the cancer registration system and the Registrar General (Scotland). Death records were complete until 1 December 2010, which served as the censor date. Cancer-specific survival was measured from the date of surgery until the date of death from colorectal cancer. The authors confirm that this study was approved by the West of Scotland Research Ethics Committee, Glasgow with written informed consent obtained from all participants.
lymphocyte infiltration (P<0.05) and plasma cell infiltration (P<0.01) were significantly associated with cancer-specific survival. The Kaplan–Meier survival curves demonstrating these relationships are shown in Figure 2.

K–M grade, lymphocyte infiltration and plasma cell infiltration were then entered into a multivariate survival model with standard clinical and pathological variables (Table 3). This demonstrated that systemic inflammatory response (mGPS; HR 2.27, P<0.01), TNM stage (HR 1.97, P<0.05), tumour necrosis (HR 1.54, P<0.05) and K–M grade (HR 2.38, P<0.05) were independently associated with cancer-specific survival (Table 3). When patients with node-negative disease were considered alone, only systemic inflammatory response (mGPS; HR 2.46, P<0.05) and K–M grade (HR 3.67, P<0.05) were independently associated with cancer-specific survival (data not shown).

The relationships between K–M grade, individual inflammatory cell types and patient-related variables are shown in Table 4. No relationships were observed between either K–M grade or lymphocyte infiltration and any of the patient-related variables studied, including markers of the systemic inflammatory response. There was a significant association between plasma cell infiltration and serum neutrophil count (P<0.05; Table 4).

The relationships between K–M grade, individual inflammatory cell types and tumour-related variables are shown in Table 5. There were significant relationships between K–M grade and tumour-related variables in colorectal cancer (Roxburgh and McMillan, 2011).

**DISCUSSION**

Results from the present study demonstrate that a strong infiltration of inflammatory cells in the invasive margin of colorectal tumours confers a distinct survival advantage for patients with primary operable colorectal cancer. Furthermore, although a strong overall inflammatory cell infiltration is a superior predictor of prognosis than the analysis of individual cell types, high numbers of lymphocytes and plasma cells in particular are beneficial to survival and associated with a number of favourable pathological characteristics. Taken together, these results indicate a prominent role for a coordinated adaptive immune response in the prevention of tumour progression in colorectal cancer.

A large number of previous studies, published over a 40-year period, have examined the prognostic value of inflammatory cell infiltration in colorectal cancer (Roxburgh and McMillan, 2011). Despite this volume of work, there is still no standardised method for the assessment of the local inflammatory response in colorectal tumours. This lack of consensus may be partly explained by the fact that many previous studies have concentrated on single cell types (Naito et al, 1998; Prall et al, 2004; Forssell et al, 2007), have relied on tissue microarrays (Pâgé et al, 2005; Galon et al, 2006; Salama et al, 2009) or have employed immunohistochemical
techniques (Oberg et al, 2005). Importantly, few previous studies have directly compared two different methods for assessing the local inflammatory response on full H&E-stained sections.

If such assessments are to move from experimental research into clinical practice, the technique employed must be simple, reproducible and easy to validate and incorporate into existing pathological staging systems. The present study suggests that a simplified overall assessment of peritumoural inflammation, using the K–M grade, fulfils these criteria and is a superior predictor of prognosis than an assessment of individual inflammatory cell types.

In addition to comparing the prognostic value of the methods described above, the present study also included a detailed examination of the cellular composition of the invasive margin of colorectal tumours. When all patients were considered, macrophages were the most prevalent cell type, followed closely by lymphocytes and neutrophils. When the cellular composition was re-examined in patients with a high-grade peritumoural inflammatory response, the relative proportion of lymphocytes increased whereas the proportions of neutrophils and macrophages fell. These findings suggest that such patients are mounting a coordinated inflammatory response at a local level, mediated primarily though cells associated with adaptive immunity.

The mechanisms by which a strong local adaptive immune response improves prognosis in patients with colorectal cancer are not clear. The present study found no association between an infiltration of inflammatory cells and any of the patient-related variables examined. In particular, there were no direct relationships with circulating levels of serum leukocytes or a systemic inflammatory response, as measured by the mGPS. These findings may therefore suggest a model whereby the initial stimulus for the development of a local inflammatory cell response is evoked by events within the tumour and its microenvironment (Nagtegaal et al, 2001). In the case of a nonspecific immune cell reaction, this may include local tissue damage caused directly by tumour invasion with subsequent hypoxia, cellular necrosis and the release of pro-inflammatory cytokines (Richards et al, 2011). Alternatively, a beneficial adaptive immune cell response may be triggered by altered antigenicity of the tumour cells themselves (Goedegebuure and Eberlein, 1995). Indeed, the presence of lymphocytes in the present study was associated with a number of favourable tumour characteristics including an expanding rather than infiltrative growth pattern, a feature previously reported as an independent prognostic factor in colorectal cancer (Cianchi et al, 1997). An association between intra-tumoural lymphocytes and lower levels of venous invasion has been reported previously (Pages et al, 2005), and the present results suggest this relationship also exists with lymphocyte infiltration at the invasive margin. That this finding has not been reported previously may be explained by the use of elastica staining in the present study to aid the detection of venous emboli; a technique resulting in a higher prevalence of venous invasion than seen in many previous studies (Roxburgh et al, 2009).

In contrast to cells associated with the adaptive immune response, an abundance of neutrophils or macrophages at the tumour border did not influence survival in the present cohort. Evidence regarding the prognostic value of these cell types, intimately associated with the innate immune response, has been conflicting. Although a number of studies have suggested that a strong infiltration of neutrophils (Baeten et al, 2006) and macrophages (Forsell et al, 2007) is beneficial to patients with colorectal cancer, others have reported no relationship with survival (Nagtegaal et al, 2001; Nagorsen et al, 2007). Indeed, in certain situations tumours may exploit these innate inflammatory cells to promote tumour proliferation and invasion (Pollard, 2004). Rather than reflecting a protective host response, the presence of these cell types in the tumour microenvironment then favours tumour growth and dissemination (Liotta and Kohn, 2001). However, using H&E-stained slides it is difficult to identify and assess the degree of macrophage infiltration. Further work using immunohistochemistry may be required to examine the prognostic value of tumour-associated macrophages, although there should be careful consideration of the markers to be used as some, such as CD68, may be expressed by other non-myeloid tissues in cancer specimens (Gottfried et al, 2008).

The present study has a number of limitations. The identification and classification of individual cell types on H&E-stained sections is a time consuming process, limiting patient numbers and restricting potential clinical application. However, this level of detail was required to compare the two methods and we can now confirm that a laborious examination of individual cells offers no
additional prognostic information compared with a simplified global assessment of inflammation. The present study focused only on the invasive margin and did not assess inflammatory cells within the tumour itself. The primary reason for this approach is that the tumour border is felt to represent a critical interface between pro- and anti-tumour factors (Zlobec and Lugli, 2009). Furthermore, inflammatory cells within the tumour itself are difficult to identify on H&E-stained sections and there is currently no global assessment of intra-tumoural inflammation against which to make a comparison. Nevertheless, an examination of the prognostic value of intra-tumoural inflammatory cells, using immunohistochemical techniques, is of considerable interest and will be the subject of future work.

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**Table 3** The relationships between clinicopathological characteristics and cancer-specific survival (multivariate survival analysis)

| Variable                                    | Univariate | Multivariate |
|----------------------------------------------|------------|--------------|
|                                              | HR (95% CI) | P            | HR (95% CI) | P |
| Sex (female/male)                            | 1.15 (0.60, 2.19) | 0.68 |
| Age (<64/65–74/>75 years)                    | 1.30 (0.87, 1.96) | 0.20 |
| Presentation (elective/emergency)           | 1.79 (0.55, 5.87) | 0.34 |
| Smoking (never/ex/current)                   | 1.19 (0.73, 1.95) | 0.49 |
| Anaemia (none/mild/severe)                   | 0.97 (0.60, 1.57) | 0.91 |
| Systemic inflammatory response (mGPS 0/1/2)  | 2.40 (1.55, 3.73) | <0.0001 |
| Tumour site (colon/rectum)                   | 0.72 (0.34, 1.53) | 0.39 |
| TNM stage (I/II/III)                         | 2.25 (1.24, 4.05) | 0.007 |
| Differentiation (well or mod/poor)           | 2.47 (1.12, 5.43) | 0.024 |
| Venous invasion (no/yes)                     | 2.38 (1.24, 4.54) | 0.009 |
| Tumour necrosis (absent/focal/moderate/extent) | 2.02 (1.36, 2.99) | <0.0001 |
| Character or margin (expanding/infiltrating) | 2.25 (1.16, 4.34) | 0.016 |
| Lymphocytes (high/low)                       | 1.98 (1.02, 3.86) | 0.045 |
| Plasma cells (high/low)                      | 2.99 (1.49, 5.99) | 0.002 |

**Abbreviations:** CI = confidence interval; mGPS = Glasgow Prognostic Score; TNM = tumour, node and metastasis.

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**Table 4** The relationships between K–M grade, lymphocyte infiltration, plasma cell infiltration and patient-related variables

| Variable | K–M grade (low/high) | P | Lymphocytes (low/high) | P | Plasma cells (low/high) | P |
|----------|----------------------|---|-----------------------|---|------------------------|---|
| Sex      | Male                 | 0.71 | 34/34                  | 1.00 | 32/36 |
|          | Female               | 29/33 | 31/31                  | 33/29 |
| Age (years) | <=64                  | 0.92 | 19/22                  | 0.91 | 19/22 |
|          | >75                  | 25/22 | 30/17                  | 23/24 |
| Presentation | Elective             | 0.17 | 61/61                  | 1.00 | 60/62 |
|          | Emergency            | 2/6 | 4/4                    | 5/3 |
| Smoking status | Never               | 0.28 | 21/30                  | 0.13 | 24/27 |
|          | Ex                  | 18/15 | 20/13                  | 14/19 |
|          | Current             | 11/10 | 12/9                   | 13/8 |
| Anaemia | Mild                 | 0.56 | 25/31                  | 0.41 | 30/26 |
|          | Moderate            | 8/15 | 14/9                   | 8/15 |
|          | Severe              | 15/13 | 10/18                  | 13/15 |
| Serum leukocytes (x 10^9/l) | White blood cell count | 9.38/9.63 | 0.63 | 9.3/8.88 | 0.50 |
|          | Neutrophils         | 6.85/6.5 | 0.12 | 6.8/5.8 | 0.10 |
|          | Lymphocytes         | 1.5/1.6 | 0.21 | 1.5/1.6 | 0.64 |
| Systemic inflammatory response (mGPS) | 0.50 | 0.70 | 0.16 |

**Abbreviations:** GPS = Glasgow Prognostic Score; K–M = Klintrup–Makinen.

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**Table 5** The relationships between K–M grade, lymphocyte infiltration, plasma cell infiltration and tumour-related variables

| Variable | K–M grade (low/high) | P | Lymphocytes (low/high) | P | Plasma cells (low/high) | P |
|----------|----------------------|---|-----------------------|---|------------------------|---|
| Tumour site | Colon               | 0.67 | 42/47                 | 0.09 | 40/49 |
|          | Rectum              | 21/20 | 25/16                 | 21/20 |
| T stage | T1/2                | 0.001 | 1/4                   | 0.41 | 6/9 |
|          | T3/4                | 0.32/23 | 5/96                | 6/15 |
| N stage | N0                  | 0.039 | 30/44                 | 0.72 | 38/36 |
|          | N1/2                | 33/23 | 27/29                 | 34/22 |
| TNM stage | I                   | 0.006 | 1/9                   | 0.78 | 5/5 |
|          | II                  | 29/35 | 33/31                 | 38/36 |
|          | III                 | 33/23 | 27/29                 | 34/22 |
| Differentiation | Well/ Moderate Poor | 0.72 | 53/57                 | 0.48 | 54/56 |
|          | Venous invasion | 0.008 | 35/52                 | 0.016 | 37/50 |
|          | Yes                 | 28/15 | 36/51                 | 29/14 |
| Tumour necrosis | Absent              | 0.018 | 3/7                   | 0.11 | 6/4 |
|          | Focal               | 26/33 | 26/33                 | 27/32 |
|          | Moderate            | 20/22 | 19/23                 | 23/19 |
|          | Extensive           | 13/4 | 14/3                  | 12/5 |
| Character or margin | Expanding | <0.001 | 25/53                 | 0.009 | 32/46 |
|          | Infiltrating        | 37/14 | 33/18                 | 32/19 |

**Abbreviations:** K–M = Klintrup–Makinen; TNM = tumour, node and metastasis.
In summary, the present study confirms that a simple assessment of peritumoural inflammation, using the K–M grade, has independent prognostic value in patients with primary operable colorectal cancer. The examination of individual cell types does not improve prediction of outcome but does suggest a prominent role for a coordinated adaptive immune response in the prevention of tumour progression in these patients. Taken together these findings give additional support to the prognostic significance of the local inflammatory response in colorectal cancer and to the idea that a simple overall assessment of peritumoural inflammation could be applied in clinical practice.

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Conflict of Interest

The authors declare no conflict of interest.

Inflammation in colorectal cancer

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Molecular Diagnostics