In colorectal cancer (CRC), a proportion of patients with early stage disease still die of metastatic or recurrent disease within 5 years of "curative" resection. Detection of carcinoma cells in the peripheral circulation at presentation may identify a subgroup of patients with micro-metastatic disease who may benefit from adjuvant chemotherapy or radiotherapy. Our aim was to determine the presence and clinical significance of colon carcinoma cells in peripheral blood at the time of surgery. Preoperative peripheral blood samples were collected from 94 patients with CRC and 64 patients undergoing bowel resection for benign conditions (adenoma, diverticular diseases or Crohn's colitis). Blood was also obtained from 20 normal donors not undergoing bowel surgery. Immunomagnetic beads were used to isolate epithelial cells followed by reverse transcription-polymerase chain reaction (RT-PCR) analysis of expression of cytokeratin (CK) 19, CK 20, mucin (MUC) 1 and MUC 2. Nineteen of 94 (20%) CRC patients were positive for epithelial cells in preoperative blood, including 6 with early stage disease. Kaplan-Meier survival analysis showed that detection of epithelial cells in preoperative blood was associated with reduced disease-free and overall survival (log-rank test, \( p = 0.0001 \)). Surprisingly, circulating epithelial cells were detected in 3/30 (10%) patients resected for adenoma, and in 4/34 (12%) patients resected for benign inflammatory conditions, suggesting that cells from nonmalignant colonic epithelium may also gain entrance into the bloodstream in the presence of bowel pathology. All 20 normal control bloods were negative for epithelial cells. Int. J. Cancer (Pred. Oncol.) 89:8–13, 2000.

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MATERIAL AND METHODS

Patients

Ninety-four consecutive patients admitted for potentially curative resection of primary CRC were enrolled in the study. Patients with overt metastatic disease were excluded. The median age of the patients was 72 years (range 32–99 years) with a median follow-up of 445 days (range 16–1,870 days). Tumours were staged according to the Dukes' staging system. Nonmalignant control subjects included 30 patients undergoing resection for colorectal adenoma, 34 patients undergoing resection for inflammatory bowel disease (Crohn's disease or diverticulitis), and 20 healthy controls without bowel disease, not undergoing surgery. Peripheral blood samples (20 ml) were collected in dipotassium EDTA. Fresh tumour tissue was obtained from CRC patients and snap frozen in liquid nitrogen for RNA extraction. The study was approved by the Queen Elizabeth Hospital Research Foundation.

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beth Hospital Ethics of Human Research Committee and informed consent was obtained in all cases.

Cell lines

The colon cancer cell lines SW48, SW480, HT29, LIM-2412, LIM-1215, LIM-2099, LIM-2405, LIM-1899, LIM-2463 and LIM-1863 were tested by RT-PCR for expression of the four epithelial-specific markers. Positive cell lines were used as controls and to optimise the RT-PCR for each of the markers. The LIM cell lines were kindly provided by Dr R. Whitehead (Ludwig Institute for Cancer Research, Melbourne, Australia); the remainder were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Cell lines were maintained in RPMI-1640 medium with 10% fetal calf serum (FCS).

RNA extraction

RNA was extracted from tissue samples and cell lines using TRI reagent (Sigma, St. Louis, MO) according to the manufacturer’s protocol.

Design of primers

The primers for CK-19 were designed to avoid amplification of the pseudogene (Datta et al., 1994) and do not amplify a product from genomic DNA at the annealing temperature used (68°C) as previously determined (Eaton et al., 1997). Primers for CK 20, MUC 1 and MUC 2 were designed to span introns thus avoiding amplification from genomic DNA. All primer pairs were checked previously determined (Eaton et al., 1997). Primers for CK-20, MUC-1 and MUC-2 were designed to span introns thus avoiding amplification from genomic DNA. All primer pairs were checked previously determined (Eaton et al., 1997). Primers for CK-20, MUC-1 and MUC-2 were designed to span introns thus avoiding amplification from genomic DNA. All primer pairs were checked previously determined (Eaton et al., 1997). Primers for CK-20, MUC-1 and MUC-2 were designed to span introns thus avoiding amplification from genomic DNA. All primer pairs were checked previously determined (Eaton et al., 1997). Primers for CK-20, MUC-1 and MUC-2 were designed to span introns thus avoiding amplification from genomic DNA. All primer pairs were checked previously determined (Eaton et al., 1997). Primers for CK-20, MUC-1 and MUC-2 were designed to span introns thus avoiding amplification from genomic DNA. All primer pairs were checked previously determined (Eaton et al., 1997). Primers for CK-20, MUC-1 and MUC-2 were designed to span introns thus avoiding amplification from genomic DNA. All primer pairs were checked previously determined (Eaton et al., 1997). Primers for CK-20, MUC-1 and MUC-2 were designed to span introns thus avoiding amplification from genomic DNA. All primer pairs were checked previously determined (Eaton et al., 1997). Primers for CK-20, MUC-1 and MUC-2 were designed to span introns thus avoiding amplification from genomic DNA. All primer pairs were checked previously determined (Eaton et al., 1997). Primers for CK-20, MUC-1 and MUC-2 were designed to span introns thus avoiding amplification from genomic DNA. All primer pairs were checked previously determined (Eaton et al., 1997). Primers for CK-20, MUC-1 and MUC-2 were designed to span introns thus avoiding amplification from genomic DNA. All primer pairs were checked previously determined (Eaton et al., 1997).

Immunobead RT-PCR

The technique for immuno-magnetic isolation of epithelial cells from blood has been described in detail previously (Hardingham, 1998). Briefly, each 10-mL blood sample was incubated with 3 × 10⁶ immuno-magnetic beads (Dynal, Oslo, Norway), labeled with monoclonal antibodies specific for different epithelial markers. Positive cell lines were used as controls and to optimise the RT-PCR for each of the markers. The LIM cell lines were kindly provided by Dr R. Whitehead (Ludwig Institute for Cancer Research, Melbourne, Australia); the remainder were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Cell lines were maintained in RPMI-1640 medium with 10% fetal calf serum (FCS).

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CK 19, CK 20 or mucin transcripts in some tumours and cell lines, despite successful amplification of other markers using the same cDNA, indicated that a panel of markers was necessary. Appropriate cell lines served as positive controls in individual RT-PCR assays.

**Sensitivity experiments**

In spiking experiments we were able to detect colon cancer cell-line cells at the level of 10 cells per 10-mL blood sample (1 cell per mL, equivalent to 1 cell per 5–10 × 10⁶ white cells). A representative result is shown in Figure 1.

**Immunobead RT-PCR analysis of blood samples**

Nineteen of 94 (20%) CRC patients were positive for epithelial RT-PCR markers in peripheral blood taken before surgical resection for malignancy. A representative result is shown in Figure 2. Two of the 19 patients were staged as Dukes’ A, 4 as Dukes’ B and 13 as Dukes’ C (Table II). Among adenoma patients 3/30 were positive, whereas 4/34 patients undergoing bowel resection for benign conditions were positive (Table II). None of 20 normal volunteers were positive for epithelial markers in blood.

**Statistical analysis**

Kaplan-Meier survival analysis of CRC patients was performed using recurrence, development of metastases or death from disease as endpoints. Data from 1 patient dying from other causes was treated as a censored observation. The log-rank test was used to compare survival curves of CRC patients positive or negative for epithelial cells in blood. There was a significant difference between the 2 groups, those positive for epithelial markers in pre-operative blood showing reduced time to relapse or death from disease.

**Figure 2** – Immunobead reverse transcription-polymerase chain reaction (RT-PCR) for cytokeratin (CK) 19: autoradiograph of controls and patient blood samples. Lane 1, positive control cDNA (LIM-1215); lane 2, positive control cDNA (LIM-2412); lane 3, negative RT control (no target); lane 4, negative PCR control (no target); lane 5, genomic DNA PCR control; lanes 6–13, patients’ preoperative blood samples. Note the positive result in lane 6 (patient 473 in Table III).

**Table II** – Patients positive for epithelial cells in preoperative blood

<table>
<thead>
<tr>
<th>Dukes’ stage</th>
<th>Number of patients</th>
<th>% Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (n = 16)</td>
<td>2</td>
<td>12.5</td>
</tr>
<tr>
<td>B (n = 47)</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>C (n = 31)</td>
<td>13</td>
<td>42</td>
</tr>
<tr>
<td>Total n = 94</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Adenoma (n = 30)</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Benign (n = 34)</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Normal (n = 18)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3** – Kaplan-Meier survival analysis: comparison of survival data from patients positive or negative for colon cells in blood: a, all colorectal cancer patients, p < 0.0001; b, Dukes’ stage C patients, p = 0.02.
The use of "nested" PCR has caused problems previously in the study. Further patients and longer follow-up is required to determine whether detection of blood-borne epithelial cells is independent of further patients and longer follow-up is required to determine whether detection of blood-borne epithelial cells is independent of tumour stage as a prognostic feature. However, within stage C patients, detection of epithelial cells in PB was independent of the extent of lymph node involvement (1–2 nodes vs. > 2 nodes, NS, Fisher’s exact test).

**DISCUSSION**

We have used the sensitive technique of immunobead RT-PCR to detect epithelial cells in peripheral blood in patients about to undergo surgical resection for colorectal malignancy or benign bowel conditions. The prior immunobead isolation of epithelial cells from blood enhances both the sensitivity and specificity of RT-PCR detection and alleviates the need to use a two-stage protocol to attain sensitivity which may result in detection of very low level "illegitimate" transcription (Chelly et al., 1989). The use of "nested" PCR has caused problems previously in assessing the specificity of CK 19 as a marker for epithelial cells (Krismann et al., 1995; Schoenfeld et al., 1994) but could be overcome by using a one-stage PCR (Battaglia et al., 1998; Eaton et al., 1997; Schoenfeld et al., 1994).

The fact that 20/20 normal control blood samples were negative by immunobead RT-PCR for all 4 epithelial markers after Southern blot hybridisation and autoradiography, and that “spiked” colon cancer cells were detectable at the level of 1 cell per 5–10^6 white blood cells, indicates that this technique is both sensitive and specific for epithelial cells. Whereas each of the markers appears to be expressed in primary tumours and colon cancer cell lines with about the same frequency, it is apparent that not all are expressed at a similar level in any one tumour (Table III), emphasising the need to use a panel of markers to avoid false-negative results.

In our study of CRC patients, the presence of epithelial-marker positive cells in preoperative peripheral blood correlated with reduced disease-free survival (p < 0.0001), suggesting that at least some of the cells detected were metastatic colon carcinoma cells in transit in the circulation. Only a very small percentage of carcinoma cells that intravasate into the bloodstream are able to form metastases since most succumb to host immunological responses or entrapment in the microvasculature (Weiss, 1990).

Among adenoma patients, 3/30 (all 3 diagnosed with large tubular adenomas) were positive in preresection blood. One of these patients (896, Table III) also had a needle biopsy of the prostate performed on the day before blood collection, so that the presence of CK 19 and CK 20 positive cells in the peripheral blood might be explained by the biopsy procedure (transrectal) with or without the release of prostatic epithelial cells.

Adenomatous tissue may contain foci of cells that have progressed toward a more malignant phenotype that may spontaneously shed cells into the circulation. However, 4/34 patients with benign inflammatory conditions were also positive in preoperative blood; the pathology in all 4 cases was described as diverticulitis; in 1 of these patients there was severe inflammation and in another, disease (p < 0.0001, hazard ratio 3.9, 95% CI 2.99–21.34) (Fig. 3a).

There were 2 Dukes’ A patients who were positive for colon cells in preoperative blood; 1 died of postoperative complications at day 54, while the other was alive and disease free at last follow-up (day 558). The 4 positive Dukes’ B patients remain disease-free at median day 391 post-surgery (range 74–604). Of 13 Dukes’ C patients in whom circulating epithelial cells were detected, 10 have died from metastatic disease, whereas another 3 are still alive following development of metastases. Kaplan–Meier survival analysis for stage C patients showed a significant survival advantage for patients who were negative for circulating epithelial cells prior to surgery, compared with patients who were positive (p = 0.02, hazard ratio 2.8, 95% CI 1.169–6.716) (Fig. 3b). Recruitment of further patients and longer follow-up is required to determine whether detection of blood-borne epithelial cells is independent of tumour stage as a prognostic feature. However, within stage C patients, detection of epithelial cells in PB was independent of the extent of lymph node involvement (1–2 nodes vs. > 2 nodes, NS, Fisher’s exact test).
ulceration and perforation of the bowel wall. Perhaps inflammatory changes and ulceration provide a means whereby normal colonic mucosal cells gain access to the bloodstream.

Angiogenesis has been found to facilitate entry of tumour cells into the circulation and has been found to be a predictor of recurrence and survival in patients with early stage breast (Weidner et al., 1992) and colon cancer (Frank et al., 1995). Tumour-associated angiogenesis has also been found to occur in adjacent normal mucosa (Fox et al., 1998) and may thus provide a mechanism whereby normal colonic epithelial cells gain access to the bloodstream. This mechanism may also be relevant in the cases of large tubular adenoma in which angiogenesis was present, as assessed by CD31 immunocytochemical staining (data not shown).

Interestingly, the frequency of detection of circulating epithelial cells in stage A or B patients was similar to that in patients with adenoma or inflammatory bowel disease (Table II). None of the 5 early stage patients with circulating epithelial cells who were followed have relapsed (Table III). Perhaps the cells detected were normal colonic mucosal cells or tumour cells without metastatic potential that had gained entry to the bloodstream via the newly formed tumour-associated vasculature.

Few studies have correlated the finding of colon tumour cells in peripheral blood with prognosis. Our earlier study of 27 patients with K-ras mutation-positive tumours showed that patients in whom circulating tumour cells were detected had a much poorer prognosis than patients who were negative (p = 0.0001) (Hardingham et al., 1995). A study of 37 CRC patients, using CK 20 as a marker for tumour cells in bone marrow and blood, found significantly reduced survival in the bone marrow-positive group (regardless of the blood status) compared with the group negative in both compartments (Soeth et al., 1997). Another study used RT-PCR to detect carcinoembryonic antigen (CEA) mRNA in peripheral blood samples from patients with cancer, including 27 with CRC. Follow-up and survival analysis showed that the frequency of cancer recurrence was significantly greater in CEA mRNA-positive patients (Mori et al., 1998). Our present results show, in a larger group of 94 CRC patients, that the presence of epithelial marker-positive cells in peripheral blood before surgical resection is predictive of shortened time to recurrence and overall survival (p < 0.0001).

Previously, epithelial-specific markers were considered appropriate for analysing preoperative blood for the presence of disseminated tumour cells as there was no published evidence to suggest that normal colon cells have access to the bloodstream in the absence of colorectal resection. We have now shown that nonmalignant colonic mucosal cells may be shed into the circulation in patients with adenoma or diverticulitis. It is feasible, therefore, that normal colonic mucosal cells may also be circulating in CRC patients and thus the finding of blood-borne epithelial cells in individual CRC patients needs to be interpreted with caution.

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