Loss of cell-cell and cell-matrix adhesion molecules in colorectal cancer

A.K. Nigam1,2, F.J. Savage1, P.B. Boulos1, G.W.H. Stamp2, D. Liu3 & M. Pignatelli2

1Department of Surgery, Rayne Institute, University College London, 5 University Street, London WC1E 6JJ; 2Department of Histopathology, Royal Postgraduate Medical School, Du Cane Road, London W12 ONN, UK.

Summary Adhesion molecules are thought to play a vital role in the induction and maintenance of tissue differentiation and their loss or down-regulation has been implicated in the neoplastic process. Recent studies have shown that the morphoregulatory activities are a consequence of interactive processes between several cell adhesion molecules rather than the function of a single molecule. Therefore, we have investigated a panel of adhesion molecules including members of the integrin, cadherin and immunoglobulin superfamily in colorectal cancer. Twenty-eight consecutive colorectal adenocarcinomas were stained using an avidin-biotin indirect immunoperoxidase technique. Our results showed a consistent loss of the &alpha;2 and &beta;1 integrin subunits (21/28 = 75% and 22/28 = 78.6% respectively) and a decrease in expression of E-cadherin in 5/5 poorly differentiated adenocarcinomas. Carcinoembryonic antigen expression was preserved but with basolateral accentuation seen in tumours. There was no statistical correlation with Dukes' stage. These results provide further evidence that in colorectal cancer there is a widespread deregulated expression of cell-cell and cell-matrix adhesion molecules. Changes in the expression and function of adhesion molecules which regulate growth and differentiation may play a role in the behaviour of colorectal cancer.

Cell-cell and cell-matrix interactions play an essential role in the induction and maintenance of a differentiated epithelial cell phenotype. Research into the process of tissue differentiation from an embryological viewpoint has received much attention. More recently developmental biology has provided us with many concepts which are applicable to neoplastic transformation, tumour invasion and metastasis. Such similarities have led us to believe that carcinogenesis may result as a disruption of normal homeostasis and structure whose control is both hierarchical and dynamic. Intercellular and cell-substratum interactions mediated by adhesion molecules are likely to play a part both in the structural morphology and functional differentiation of the tissue and therefore a loss in this control mechanism may well facilitate the neoplastic process (Crossin, 1991).

There are four main groups of adhesion molecules, integrins, cadherins, the immunoglobulin superfamily and lecans (selectins) (Hynes & Lander, 1992). Integrins and cadherins are the prime mediators of cellular adhesion in normal and transformed epithelial cells, integrins being largely responsible for cell-substratum interaction and cadherins for intercellular interaction (Hynes, 1992; Takeichi, 1991).

Integrins are heterodimers composed of &alpha; and &beta; subunits which are non-covalently bound. They are transmembrane receptors whose function is dependent on the presence of both subunits. At least 14&alpha; and 8&beta; subunits have been described with 20 combinations (Hynes, 1992). The &beta;1 subfamily (Very Late Antigens—VLA) associates with one of at least eight different &alpha; chains to form receptors for extracellular matrix proteins including fibronectin, laminin and collagen (Hemler, 1990). &beta;1 integrins have been shown to be important in the glandular differentiation of a colorectal cancer cell line (Pignatelli et al., 1992a).

The &beta;3 and &beta;5 subunits are more selective in their associations with &alpha; chains and form receptors for multiple ligands such as vitronectin, fibrinogen and collagen. &alpha;v links preferentially with &beta;3, but may also associate with &beta;1, &beta;3 or &beta;5. However, there is some evidence to suggest an alteration in &alpha;v affinity in malignant tissue towards &beta;5 (Korhonen et al., 1992).

Cadherins are transmembrane adhesion molecules that require calcium for their function and connect cells in a homotypic fashion (Takeichi, 1991). They are the prime mediators of intercellular interaction, so much that inactivation of other cell-cell adhesion molecules has little effect on cadherin function (Duband et al., 1987). Several subclasses of cadherins have been described including E-cadherin (epithelial cadherin, L-CAM, uvomorulin, Arc-1, cell CAM 120/80), P-cadherin (placental) and N-cadherin (neural). E-cadherin is expressed by normal epithelial cells (Shiozaki et al., 1991) and has also been implicated as a major determinant in the differentiation of a colorectal carcinoma cell line (Pignatelli et al., 1992a).

The immunoglobulin superfamily of adhesion molecules consists of members such as carcinoembryonic antigen (CEA) and the neural cell adhesion molecule (N-CAM). N-CAM has been shown to have homology with the DCC gene (deleted in colorectal carcinoma), which is thought to be important in colorectal tumorigenesis (Fearon et al., 1990). CEA appears to function as an adhesion molecule both mediating intercellular as well as cell-matrix interactions (Benchimol et al., 1989; Pignatelli et al., 1990b).

We have investigated the immunolocalisation of a panel of eight adhesion molecules in colorectal cancer and correlated our findings to Dukes' stage and morphological differentiation. The panel has been selected to include members from the three main families of cell adhesion molecules responsible for epithelial cell interactions as discussed above and which have been shown to be expressed in a range of epithelial tissues. We also discuss the possible functional interaction between the molecules studies as there is now overwhelming evidence to support the theory that the biological behaviour of tumour cells is the manifestation of a composite of multiple adhesion interactions (Hynes & Lander, 1992).

Materials and methods

Surgical resection specimens of 28 colorectal adenocarcinomas were obtained. Biopsies from the tumour centre, the tumour-normal mucosal junction and normal colonic mucosa 10 cm distant from the tumour were taken. These were immediately mounted in gelatin and snap-frozen in liquid nitrogen. They were stored at −20°C until sectioning. Cryostat sections of 6 μm thickness were cut, air dried and fixed in cold 50% acetone/methanol for 10 min prior to staining. Non-specific binding was reduced by pre-incubation with 20% normal rabbit serum for 15 min. An indirect avidin-biotin immunoperoxidase technique was employed, using the primary monoclonal antibodies shown in Table I. DH12, HAS-6 and &beta;5 were obtained in purified form and used at 20 μg/ml concentration in phosphate buffered saline (PBS). MP4F10, PR3B10, 13C2, 23C6 and HEC-1 were obtained.

Correspondence: M. Pignatelli.
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as culture supernatant and used neat. All the antibodies used were in saturating concentrations determined from previous immunohistochemical studies (Pignatelli et al., 1992; Stamp & Pignatelli, 1991). 4-well Multiwell glass slides (C.A. Hendley, Ltd.) pre-treated with 0.1% poly-l-lysine solution 1:10 were used for the immunostaining. Briefly, after pre-incubation the primary antibody was placed on each section for 45 min at room temperature. After three washes in PBS, the second antibody, rabbit anti-mouse immunoglobulins labelled with biotin in a mixture with normal human serum was added for 30 min. After three further washes in PBS the sections were incubated with the avidin-biotin complex (Dakopatts, High Wycombe, UK) for another 30 min. Following three further washes in PBS, diaminobenzidine (Sigma, St Louis, MO) was used to visualise the horseradish peroxidase activity and the sections were then counterstained with haematoxylin and dehydrated in a graded alcohol series.

After clearing with xylene, the sections were mounted in DPX (British Drug Houses, Dagenham, Essex, UK). In control sections either the second or third layer alone was used with omission of the primary antibody and no specific staining was observed in these cases. The positive controls were the corresponding normal tissue itself, thus allowing direct comparison to be made with tissue taken from the same patient at the same time. Intensity of staining was then assessed by two different observers (A.K.N., M.P.) on two separate occasions using light microscopy and scored as follows: + + + (uniform and strong), + + (moderate), + (weak and patchy) and − (negative). These were scored as 3, 2, 1 and 0 for purposes of statistical evaluation. Photomicrographs were taken on an Olympus PM10 ADS system.

The histological diagnosis, grade, recorded as well, moderate and poorly differentiated assessed by glandular morphology and classification according to Dukes' stage, were based on assessment of corresponding tissue removed for conventional analysis. Adhesion molecule expression, i.e., intensity of staining was then correlated with tumour grade and stage. The $\chi^2$ test with Yates' correction was employed for this analysis. The numerical differences in expression between normal and tumour tissue were also ranked and the Mann-Whitney U test applied to the data.

Results

Twenty-eight consecutive cases of colorectal carcinoma resected by one surgeon (P.B.B.) were used in this study. Their clinicopathological characteristics were as shown in Table II. There was a comparable number of metastatic and non-metastatic cases (Dukes' A + B = 15, C = 13) but there was a preponderance of cases in the moderately differentiated category.

Normal colonic epithelium showed a similar pattern of staining with the $\beta_1$, $\alpha_2$, and $\alpha_6$ antibodies (Figure 1). There was strong membrane and cytoplasmic staining observed in all cases as well as strong endothelial immunoreactivity. In

### Table I Monoclonal antibodies

| Monoclonal antibody | Antigen recognised | Reference |
|---------------------|--------------------|-----------|
| DH12                | $\beta_1$ integrin subunit | De Strooper et al., 1988 |
| HAS-6               | $\alpha_2$ integrin subunit | Tenchini et al., 1993 |
| 13C2                | $\alpha_4$ integrin subunit | Davies et al., 1989 |
| 23C6                | $\alpha_3$$\beta_3$ integrin | Davies et al., 1989 |
| $\beta_5$           | $\beta_5$ integrin subunit | Ramaswamy & Hemler, 1990 |
| MP4F10              | $\alpha_6$ integrin subunit | Pignatelli et al., 1992b |
| PR3B10              | CEA/NCA             | Richman & Bodmer, 1987 |
| HEC1                | E-cadherin          | Shimoyama et al., 1989 |

**Abbreviations:** CEA, carcinoembryonic antigen; NCA, non-specific cross-reacting antigen; E-cadherin, epithelial specific cadherin.

### Table II Clinicopathological characteristics

| Patient | Age | Sex | Site of tumour | Differentiation | Dukes' stage |
|---------|-----|-----|----------------|-----------------|--------------|
| 1       | 48  | F   | Rectum         | Moderate        | C            |
| 2       | 57  | F   | Desc. colon    | Moderate        | C            |
| 3       | 72  | F   | Asc. colon     | Moderate        | B            |
| 4       | 60  | F   | Rectum         | Moderate        | C            |
| 5       | 61  | F   | Rectum         | Moderate        | B            |
| 6       | 48  | M   | Sigmoid        | Moderate        | C            |
| 7       | 60  | M   | Desc. colon    | Moderate        | C            |
| 8       | 60  | F   | Rectum         | Moderate        | B            |
| 9       | 79  | F   | Rectum         | Poor            | C            |
| 10      | 80  | F   | Sigmoid        | Moderate        | B            |
| 11      | 64  | M   | Desc. colon    | Moderate        | B            |
| 12      | 72  | F   | Desc. colon    | Moderate        | A            |
| 13      | 69  | M   | Rectum         | Moderate        | B            |
| 14      | 89  | M   | Desc. colon    | Moderate        | B            |
| 15      | 75  | F   | Asc. colon     | Moderate        | B            |
| 16      | 86  | F   | Rectum         | Poor            | C            |
| 17      | 78  | F   | Asc. Colon     | Moderate        | B            |
| 18      | 62  | M   | Caecum         | Moderate        | B            |
| 19      | 91  | M   | Desc. colon    | Moderate        | C            |
| 20      | 87  | M   | Sigmoid        | Moderate        | B            |
| 21      | 75  | M   | Desc. colon    | Poor            | C            |
| 22      | 34  | M   | Asc. colon     | Well            | C            |
| 23      | 70  | F   | Rectum         | Moderate        | C            |
| 24      | 72  | M   | Desc. colon    | Poor            | C            |
| 25      | 68  | F   | Rectum         | Moderate        | A            |
| 26      | 62  | M   | Sigmoid        | Moderate        | A            |
| 27      | 75  | M   | Asc. colon     | Poor            | C            |
| 28      | 68  | M   | Rectum         | Well            | B            |

**Figure 1** Normal and tumour tissue stained for $\alpha_2$ subunit showing a down-regulation of expression in a moderately differentiated carcinoma. (bar = 50 μm).
addition, β1 was present in fibroblasts and smooth muscle. E-cadherin also demonstrated a distinct and evenly distributed expression at the intercellular borders and the apical surfaces of the epithelial cells. Carcinoembryonic antigen (CEA) staining was uniform with luminal staining observed in all cases. In contrast, however, antibodies to αv, αvβ3 and β5 showed marked differences in their staining patterns in normal colonic epithelium. The αv was expressed strongly in endothelial tissues but weakly on the epithelium. Its reactivity in the tissue stroma was much more predictable being present in all cases except two. The αvβ3 complex was absent from the epithelium in 17/28 cases and showed only weak immunoreactivity in the remainder. Its presence in the normal tissue stroma essentially matched that of the αv subunit. β5 was weakly and inconsistently expressed in the stroma and the epithelium (Figure 2).

The differences in staining observed between normal and tumour tissue are shown in Tables III and IV. Although many tumours exhibited heterogeneity of expression, some clear patterns emerged. There was a consistent reduction in expression of the α2 and β1 subunits with some cases showing a complete loss (Figure 3). 75% and 78% of tumours respectively showed a down-regulation (Figure 4). However, neither of these results reached statistical significance when analysed by Dukes’ stage ($\chi^2 = 3.59, \ p = 0.06 \ \chi^2 = 0.44, \ \ P = 0.50$). Furthermore, after ranking the data in terms of degree of down-regulation, again the figures failed to reach significance ($P = 0.43$ and $P = 0.45$ respectively, Mann-Whitney U test). Decreased immunoreactivity was observed only in poor and moderately differentiated tumours. However, our sample size in the well differentiated category was small and we are therefore unable to infer any conclusions regarding a possible progressive loss of expression with worsening differentiation.

E-cadherin exhibited decreased immunoreactivity in 8/28 tumours. No correlation with Dukes’ stage was found ($\chi^2 = 3.32, \ P = 0.07$) even after ranking level of expression ($P = 0.43$). All the five poorly differentiated carcinomas showed a loss of expression whereas in 20/23 well or moderately differentiated tumours expression was preserved (Figure 5).
α6 expression was essentially preserved (Figure 6) with a minimal reduction in expression (+ + + to + +) seen in five tumours. CEA was down-regulated minimally in only 3/28 tumours with good luminal immunoreactivity (Figure 7) but also a polarisation to the basolateral aspect of the cell. Neither of these results corresponded with Dukes' stage or morphological differentiation.

The greatest variability in expression was observed in the stroma for αv, αvβ3 and β5 subunits. (Table V). In the tumour epithelium there was little change with weak heterogeneous expression observed in those cases where it had been noted in the corresponding normal tissue. However, stromal αv was reduced in 5/28 cases (Figure 8) with an increase in expression in four tumours. αvβ3 was downregulated in ten cases (Figure 9) with a slight increase in expression seen in only one tumour. β5 was lost from the stroma in four cases but showed an increase in immunoreactivity in eight cases (Figure 10). The overall results in this group of integrins (Figure 11) therefore suggests a loss of the αvβ3 complex from the tumour stroma with an increase in the β5 subunit. As αv loss occurred in only 17% of tumours, this implies that the αv retained may be exhibiting a preferen-
Table V Stromal expression of αv, αvβ3 & β5

| Patient | αv | T | αv, β3 | T | β5 | T |
|---------|----|---|--------|---|----|---|
| 1       | +  | + | -      | - | -  | - |
| 2       | ++ | + | +      | + | +  | + |
| 3       | +  | + | +      | + | ++ | + |
| 4       | ++ | + | ++     | + | ++ | + |
| 5       | ++ | + | +      | - | -  | - |
| 6       | ++ | + | +      | - | -  | - |
| 7       | +  | - | -      | - | +  | + |
| 8       | -  | - | -      | - | +  | + |
| 9       | +  | + | +      | + | +  | + |
| 10      | ++ | + | +      | + | +  | + |
| 11      | ++ | + | +      | + | +  | + |
| 12      | ++ | + | +      | + | +  | + |
| 13      | ++ | + | +      | + | +  | + |
| 14      | ++ | + | +      | + | +  | + |
| 15      | ++ | + | +      | + | +  | + |
| 16      | ++ | + | +      | + | +  | + |
| 17      | ++ | + | +      | + | +  | + |
| 18      | ++ | + | +      | + | +  | + |
| 19      | ++ | + | +      | + | +  | + |
| 20      | ++ | + | +      | + | +  | + |
| 21      | ++ | + | +      | + | +  | + |
| 22      | ++ | + | +      | + | +  | + |
| 23      | ++ | + | +      | + | +  | + |
| 24      | ++ | + | +      | + | +  | + |
| 25      | ++ | + | +      | + | +  | + |
| 26      | ++ | + | +      | + | +  | + |
| 27      | ++ | + | +      | + | +  | + |
| 28      | ++ | + | +      | + | +  | + |

Statistically, there was no correlation with Dukes' stage with reduced or increased expression for the αv (χ² = 3.59, p = 0.06 and χ² = 2.85, P = 0.11) and the β5 subunits (χ² = 0.44, p = 0.5 and χ² = 3.31, P = 0.07). The same result was gained after a ranking analysis. No pattern relating to differentiation was discernible in this subgroup.
Discussion

The importance of cellular adhesion in the progression of a malignant neoplastic process has long been recognised. Over 50 years ago it was proposed that a loss of intercellular adhesion between tumour cells might be an important factor in the spread of cancer. Fidler and Hart (1982) also concluded that the ability to infiltrate surrounding tissues and subsequently to detach and migrate may be related to alterations in adhesiveness between cells and between cells and their surrounding matrix. Furthermore, once the tumour cells have entered the vasculature or lymphatic system their ability to form metastasis may also be related to relative changes in adhesion receptor expression. It has also been shown that morphogenesis of normal and transformed cells is, in part, governed by the functional expression of these molecules. In particular, the beta-1 integrins and E-cadherin have been shown to have regulatory properties over the differentiation of a colorectal carcinoma cell line (Pignatelli et al., 1992a). Although the functional cooperation between these molecules is poorly understood, what is apparent is that the full evolution of any biological process involving adhesion molecules is likely to involve a multitude of receptor-ligand interactions (Hynes, 1992).

With the above in mind, we studied a panel of adhesion molecules with a view to identifying those receptors which were aberrantly expressed in the colonic neoplastic process. We found that the beta1 subunit was consistently lost or decreased in the moderately and poorly differentiated carcinomas. In the same tumours the a2 subunit was similarly affected. The a2beta1 receptor binds to collagens, laminin and fibronectin depending on the cell type (Kirchhofer et al., 1990). The loss of this integrin in poorly differentiated colorectal carcinomas has been previously reported by one of the authors (Pignatelli et al., 1990a), but studies in other tumours and cell lines have reported different findings. Koretz and co-workers (1991) found a marked heterogeneity of expression for this integrin even in normal colonic mucosa with a complete loss of antigen expression in two tumours. No such loss has been seen in pancreatic cancer (Weinelt et al., 1992) and a role in tumour invasion has been implicated by Chan et al. (1991) in their study on rhabdomyosarcoma cells in which overexpression of a2beta1 led to an increased metastatic potential. This may be a reflection on the different ligand specificities of a2beta1 already referred to or the differences in epitopes recognised by the monoclonal antibodies used in the various studies. As no correlation to Dukes' stage was found in our study, inferences on the role played by this integrin in invasion and metastasis are difficult to make. The important point to note is that this was a selective integrin loss as the a6 subunit was almost entirely preserved. This latter finding is not in keeping with studies on breast carcinomas nor renal cell carcinomas which show a loss of this subunit in tumours where there is a high degree of loss or disturbance of basement membrane (Pignatelli et al., 1992b; Korhonen et al., 1992). The a6beta1 integrin is a receptor for the E8 fragment of basement membrane laminin (Lotz et al., 1990), and in view of our apparent contradictory findings for these two subunits, it follows that in colorectal carcinoma a6 complexes with another beta subunit. There is strong evidence to support the preferential association of a6 with beta4 in normal and transformed epithelial cells (Sonnenberg & Linders, 1990; Lee et al., 1992) and we suggest that in the colon this may well be the case.

The role of integrins in the loss of differentiation that occurs in most tumours is well-established. Members of the beta integrin subfamily have been implicated in the process of tubule formation in colonic epithelium and recently, functional studies using a specific monoclonal antibodies in colon carcinoma cell lines have identified the a2beta1 heterodimer as the key mediator of this process (Pignatelli & Liu, personal communication). Thus, the loss of a2beta1 may explain, at least in part, the disturbance of cell polarity and glandular organisation seen in poorly differentiated colorectal adenocarcinomas.

![Figure 10](image_url)  
**Figure 10** Beta5 overexpression in the stroma of a moderately differentiated carcinoma. (bar = 50 μm).

![Figure 11](image_url)  
**Figure 11** Stromal changes in expression of αv, αvβ3 & β5.
In this paper we report for the first time the distribution of \( \alpha v, \beta 3 \) and the heterodimer \( \alpha v \beta 3 \) in colorectal cancer. \( \alpha v \beta 3 \) is the classical vitronectin receptor and elevated expression of this integrin has been associated with invasive melanoma in vitro (Felting-Habermann et al., 1992) and in vivo (Albelda et al., 1990). Treatment of melanoma cells with an antibody to the integrin causes an increase in their ability to invade basement membrane matrices concomitant with an increase in expression of a matrix degrading enzyme, 72 kDa gelatinase, both at the mRNA and protein level (Seftor et al., 1992). Furthermore, \( \alpha v \) appears to be the important subunit responsible for this finding. Our results suggest a stromal distribution for the \( \alpha v \beta 3 \) receptor in colorectal tissue. A moderate proportion (35%) of tumours showed decreased expression of \( \alpha v \beta 3 \) in the stroma, but with preservation of the \( \alpha v \) subunit immunoreactivity. Fewer tumours lost this subunit and in our case there was no increase in expression. The increase in immunoreactivity observed with \( \beta 5 \) suggests an altered affinity of the \( \alpha v \) subunit. There appears to be a preferential association of \( \alpha v \) with the \( \beta 5 \) subunit in malignant tissue and a corresponding reduction in expression of the \( \alpha v \beta 3 \) complex. Breast and renal cell carcinomas show a similar association (Pignatelli et al., 1992b; Korhonen et al., 1992). It is tempting to speculate that the stromal localisation of the \( \alpha v \beta 3/\alpha v \beta 5 \) integrins may have a controlling influence over the production and activity of metalloproteinases which would aid in the enzymatic digestion of the extracellular matrix. There is an increasing body of evidence to support the stromal cells as being the source of these enzymes (Poulson et al., 1992) and thus the nearby localisation of these particular integrins would imply an integral role. This would be in keeping with the results seen in melanoma cell lines (Seftor et al., 1992).

E-cadherin expression was preserved in the well and moderately differentiated carcinomas. However, in all the poorly differentiated carcinomas there was a marked decrease in expression. The same tumours also showed loss of the \( \alpha 2 \) and \( \beta 1 \) subunits. If tumours undergo a progressive loss of differentiation during their genesis and subsequent growth then it would appear from our findings that \( \alpha 2 \beta 1 \) loss is a relatively early event in the dedifferentiation process and E-cadherin loss a later occurrence. Van der Wurff (1992) reported similar findings on E-cadherin in poorly differentiated colorectal carcinomas but also suggested that there was a gradual loss of E-cadherin from adenoma to high grade carcinoma. Our results, although from comparable small numbers, do not support that theory as the decrease in expression of E-cadherin in our series was limited to the least differentiated specimens. Gastric carcinoma shows a similar distribution pattern to that which we found in colorectal carcinoma (Shiozaki et al., 1991). Matsuura et al. (1992) have shown that cells from a primary gastric tumour found in ascites show an absence of staining for E-cadherin and decreased intercellular adhesion. This latter finding suggests a role for E-cadherin in the detachment and infiltrative process. Although there was no significant correlation with metastasis in our study the functional activity of the E-cadherin expres-

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