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CD44 is Associated with Proliferation in Normal and Neoplastic Human Colorectal Epithelial Cells

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Flash-frozen biopsies obtained from surgical specimens of three adenomatous polyps and 22 colorectal adenocarcinomas (19 primary and three metastatic) were tested by immunohistochemistry for CD44 expression using F10-44-2 monoclonal antibody. CD44 positivity was correlated with proliferative status defined by Ki-67 monoclonal antibody reactivity. In normal colonic mucosa, CD44 was expressed in the proliferative zone of crypts. In tumours, CD44 expression was associated with proliferative areas irrespective of tumour stage or differentiation. Non-proliferating areas of the carcinomatous epithelium did not express CD44 although non-proliferating stromal lymphoid tissue did. There was no apparent association with tumour progression. F10-44-2-defined CD44 is consistently expressed during proliferation by normal colorectal epithelial cells and by both benign and malignant colorectal tumour cells.
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INTRODUCTION

CD44, AN ABUNDANT and ubiquitous cell adhesion molecule, has been linked with the development and spread of cancer. A distinct form of the CD44 molecule found on some epithelial cells appears to be increased by neoplastic transformation [1] and in nude mice CD44 has been associated with an increase in the number of lung metastases formed by a human melanoma cell line [2]. Furthermore, in rat pancreatic adenocarcinomas a CD44 splice variant has been indicated to confer metastatic potential when transfected into non-metastasising cells [3], and

sequences homologous to this splice variant have been found in human carcinoma cell lines [4]. In solid tumours, elevated levels of CD44 have been associated with high-grade gliomas [5] and with tumour spread and poor prognosis of large cell lymphomas [6].

CD44 was originally described as a glycoprotein surface marker of human T lymphocytes defined by monoclonal antibody F10-44-2 [7], and later found to be immunologically identical to the Hermes antigen [8] and the transmembrane receptor for hyaluronate [9, 10], amongst others [11]. A diverse range of functions has been attributed to the CD44 molecule, which is consistent with its broad tissue distribution [12]. In leukocytes CD44 is involved in adhesion to high endothelial cells and homing to peripheral lymphoid organs [13, 14]. In epithelial cells CD44 mediates anchorage to hyaluronate in basement membranes [15]. CD44 has also been shown to be directly involved in cell-to-cell adhesion [16] and to contribute to T and B cell activation [17]. This wide range of cellular functions has been partly explained by cDNA sequence data that demonstrate several different forms of CD44 obtained from the same gene by variations in splicing [18].

The functions attributed to CD44 are consistent with a role in cancer growth. For example, many of the steps in lymphocyte migration, such as entrance to and survival in the circulation, may be mimicked by CD44-expressing tumour cells. This possibility has been supported by a recent report that a CD44 variant, which confers metastatic potential to epithelial cells, shares sequences with a form transiently expressed on B and T lymphocytes [19]. The hyaluronic acid receptor function of CD44 may aid tumour growth in various ways, since the glycosaminoglycan hyaluronic acid is involved in cell motility, and angiogenesis and embryonic development [20, 21]. Furthermore, high hyaluronate levels have been detected in many tumour types [22] and carcinoma cells can stimulate production of hyaluronate by normal human fibroblasts [23].

In normal human epithelial cells [12], hamster epithelial cells [15] and CD44-transfected 3T3 cells [15], CD44 expression has been linked to proliferation. The question arises of whether the association of CD44 with human cancer is linked to proliferative status, as has been reported in these cases, or to other aspects of tumour development. To address this we used immunohistochemistry to study proliferation and CD44 distribution in normal colon mucosa and in progressive stages of colonic tumour development. The results indicate that CD44 is expressed during proliferation of human colonic epithelial cells within colorectal tumours and flanking normal tissue.

MATERIALS AND METHODS

Clinical material

Biopsies were flash-frozen from surgical specimens of adenomatous polyps (3 patients), primary colonic adenocarcinomas (19 patients) and hepatic metastases of colonic adenocarcinoma (3 patients). Adjacent histologically normal epithelial tissue was collected for 13 of the 19 adenocarcinoma cases and all three adenomatous polyps. In all cases, formalin-fixed specimens taken adjacent to the flash-frozen biopsies were independently processed and reported for routine histopathology (Department of Pathology, Charing Cross Hospital, U.K.). Differentiation grade was independently assessed on sections used for immunocytochemistry. Details of tumour stage and differentiation (grade) are shown in Table 1.

Antibodies

The mouse monoclonal antibody F10-44-2 to CD44 [7] was obtained lyophilised from British Biotechnology (Oxford, U.K.). The antibody had been purified from ascitic fluid by ammonium sulphate precipitation and ion exchange chromatography.

Ki-67 mouse monoclonal antibody to proliferating cells was obtained as dialysed tissue culture supernatant from Dako-Patts (High Wycombe, U.K.). Ki-67 reacts with a human nuclear antigen expressed on all proliferating cells in human tissues [24]. The antigen is present in S, M, G_2 and late G_1 phases of the cell cycle, and is consistently absent in G_0.

Immunohistochemistry

Frozen tissue sections (6 μm) of the unfixed biopsies were air dried and fixed in 0.4% paraformaldehyde for 20 min at 4°C. This was followed by washing in three changes of phosphate-buffered saline (PBS) [0.01 mol/l phosphate pH 7.4, 0.9% (w/v) NaCl]. Sections were then covered with 10% normal horse serum for 20 min, drained and incubated overnight at 4°C with anti-CD44. A serial section from each block was incubated with the Ki-67 antibody. After incubation overnight, sections were washed in three changes of PBS and then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody 20 μg/ml (Vector Laboratories, Peterborough, U.K.) containing 10% normal human serum. After incubation for 30 min in a moist chamber, slides were washed three times with PBS, mounted with 'Shield' (Vector Laboratories), to avoid rapid fluorescence decay, and visualised and photographed using a Zeiss Axiophot fluorescence microscope.

RESULTS

CD44 and Ki-67 expression in normal colorectum

CD44 expression was determined by immunohistochemistry on samples from 16 patients. Results in all cases showed strong staining with anti-CD44 antibody on the cytoplasm of epithelial cells in the lower part of the colonic crypt (Fig. 1a). There was a precise match with the area of cells stained with Ki-67 antibody in serial sections (Fig. 1b). Neither CD44 nor Ki-67 reactive antigen were detected in the upper parts of the normal crypts (Fig. 1a, b). Control sections without anti-CD44 and Ki-67 primary antibody showed no immunofluorescence (not shown).

Strong and homogeneous CD44 expression was detected in the lamina propria of all patients studied; an example is shown in Fig. 1a. Staining of a serial section with Ki-67 monoclonal antibody indicated that, in the lamina propria, CD44 staining was not paralleled by Ki-67 staining (Fig. 1b).

Adenomatous polyps

Anti-CD44 and Ki-67 were applied to biopsies from three adenomatous polyps; patient details are shown in Table 1. These tumours were benign (two were mildly dysplastic and one was moderately dysplastic). The results showed that, in the areas studied, epithelial cells forming glandular structures stained strongly with anti-CD44. An example is shown in Fig. 1c

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Table 1. Comparison of anti-CD44 staining with stage and grade of tumours from 3 patients with adenomatous polyps and 22 patients with colorectal cancer

Patient no. and tumour	Age/sex		Dukes stage	Grade	Anti-CD44 staining
Adenomatous polyp					
1*	—	—		Moderately dysplastic	+++
2	—	—		Mildly dysplastic	++
3	—	—		Mildly dysplastic	++
Adenocarcinoma					
4*	65	M	A	WD	+++
5	69	M	A	WD	+++
6	73	F	A	MD	+
7	83	F	B	MD	+
8	83	M	B	MD	+
9*	78	M	B	MD	+++
10	75	F	B	MD	+
11	87	F	B	MD	+/−
12	70	M	B	MD	+/−
13	—	M	B	MD	++
14	62	M	B	MD	++
15	69	M	B	PD	+++
16*	65	F	C	MD	+
17	70	F	C	MD	+/−
18	83	F	C	MD	++
19	56	M	C	MD	+
20*	64	F	C	PD	+++
21	41	F	C	MD	+
22	83	F	C	PD	+++
Metastatic adenocarcinoma in liver					
23*	59	M		PD	+++
24	57	M		MD	+
25	59	M		MD	++

M, male; F, female; WD, well differentiated adenocarcinoma; MD, moderately differentiated; PD, poorly differentiated. Strong and homogeneous anti-CD44 staining is depicted by +++, moderate or patchy staining by ++, weak staining by + and just discernible staining by +/−. Patient numbers marked with an asterisk (*) are illustrated in the figures.

(adenomatous polyp, patient no. 1). This figure illustrates that anti-CD44 staining was uniform throughout the tumour epithelial cells. Serial sections to those shown in Fig. 1c were stained for proliferation using the Ki-67 antibody. The results show Ki-67 staining of the nuclei of tumour cells in areas corresponding to those with strong cytoplasmic staining for CD44 (see Fig. 1d).

Primary adenocarcinomas

Biopsies from two well differentiated, 14 moderately differentiated and three poorly differentiated colonic adenocarcinomas were studied; patient details are shown in Table 1. Results obtained with the well differentiated carcinomas showed homogeneous positive staining for CD44 in carcinoma cells and stromal lymphoid cells (Fig. 1e; patient no. 4). Ki-67 staining on serial sections showed corresponding staining of epithelial areas (Fig. 1f) but lymphoid cells stained with anti-CD44 did not react with the Ki-67 proliferation marker (Fig. 1f).

For the moderately differentiated adenocarcinomas, proliferative areas were patchy and tended to be mainly at the edge rather than the centre of tumours. CD44 was consistently expressed on epithelial cells in the proliferative areas and was absent in the non-proliferative areas. An example from patient 9 of a CD44-positive epithelial area, edged by CD44-positive lymphoid tissue and surrounding a central necrotic zone is shown in Fig. 1g. Ki-67 antibody was applied to a serial section and results indicated that the CD44-positive carcinomatous epithelium was in the process of proliferation (see Fig. 1h). However, as before, this was only true for the epithelial cells. Stromal lymphoid cells which stained with CD44 in Fig. 1g were not stained by the Ki-67 antibody (Fig. 1h). An example from patient 16 of CD44-negative tumour gland-like structures (amongst CD44-positive lymphoid tissue) is shown in Fig. 1i. These areas of tumour which did not express CD44 did not appear to be proliferating when tested with the Ki-67 cell proliferation marker on serial sections (see Fig. 1j).

The three poorly differentiated adenocarcinomas were heterogeneously positive when stained for CD44. An example is shown in Fig. 1k (poorly differentiated adenocarcinoma; patient no. 20). A serial section to that shown in Fig. 1k was stained for proliferation using the Ki-67 antibody. The results showed uniform Ki-67 staining in areas corresponding to those staining for CD44 (see Fig. 1l).

Metastatic adenocarcinomas

Three cases of hepatic metastatic adenocarcinoma were also studied with the anti-CD44 and Ki-67 antibodies. CD44 staining

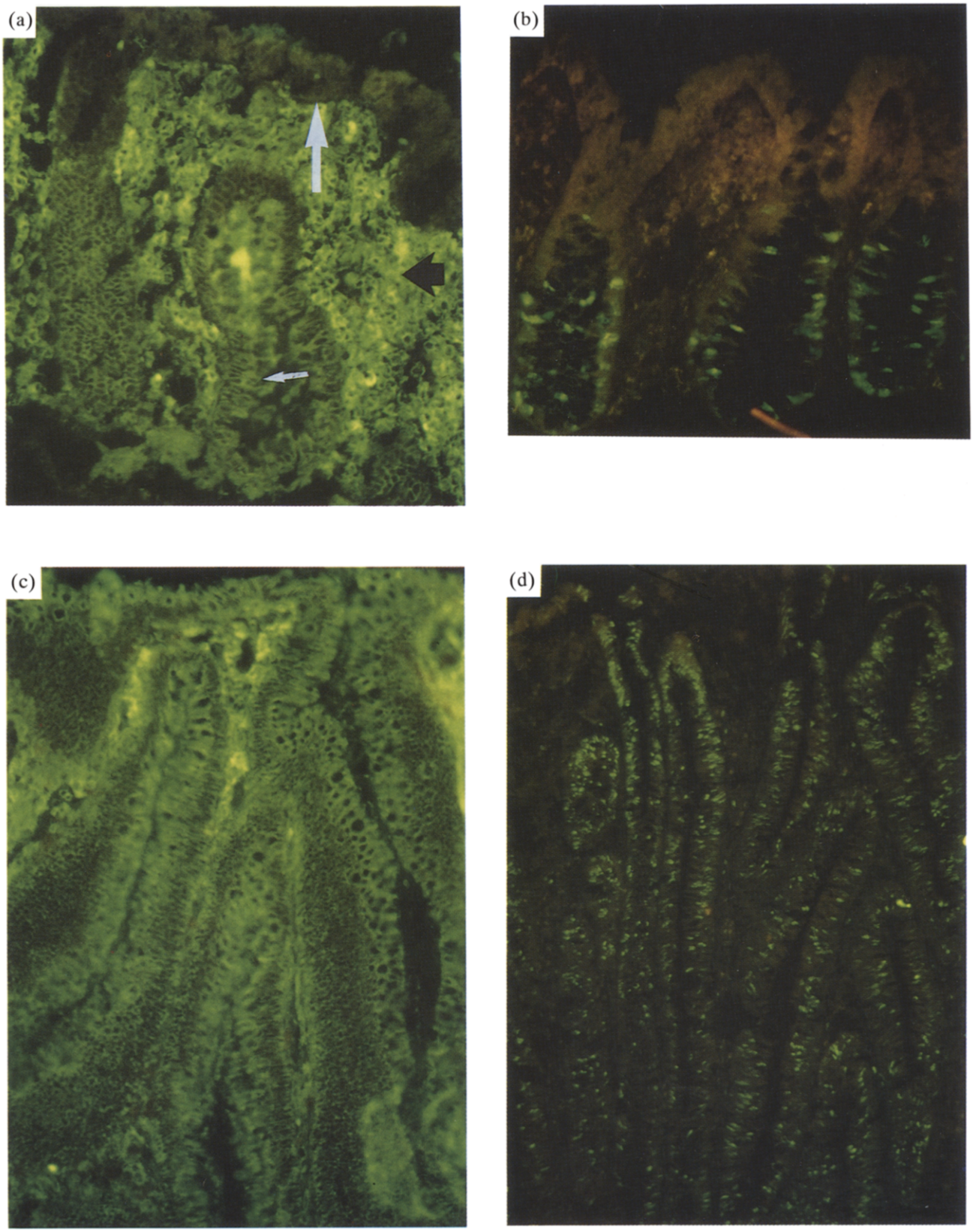


Fig. 1. Immunofluorescence staining of frozen tissue sections using antibody to the CD44 cell adhesion molecule and to the Ki-67 cell proliferation marker. Patient details are shown in Table 1. (a)___ = 30μm. CD44 staining detected in cytoplasm of epithelial cells in the lower part of normal colonic crypt (small arrow) and also in lymphoid cells of the lamina propria (black arrow), note the negative staining of upper crypt and surface epithelium (large arrow). (b)___ = 30μm. Nuclear staining for Ki-67 on a serial section to (a). Ki-67 staining complemented that of CD44 on epithelial cells but did not detect cells in the lamina propria. (c)___ = 30μm. Adenomatous polyp patient no.1; strong CD44 staining is detected homogeneously in epithelial cells. (d)___ = 30μm. Serial section to (c), staining for Ki-67 is homogeneously positive on epithelial cells.

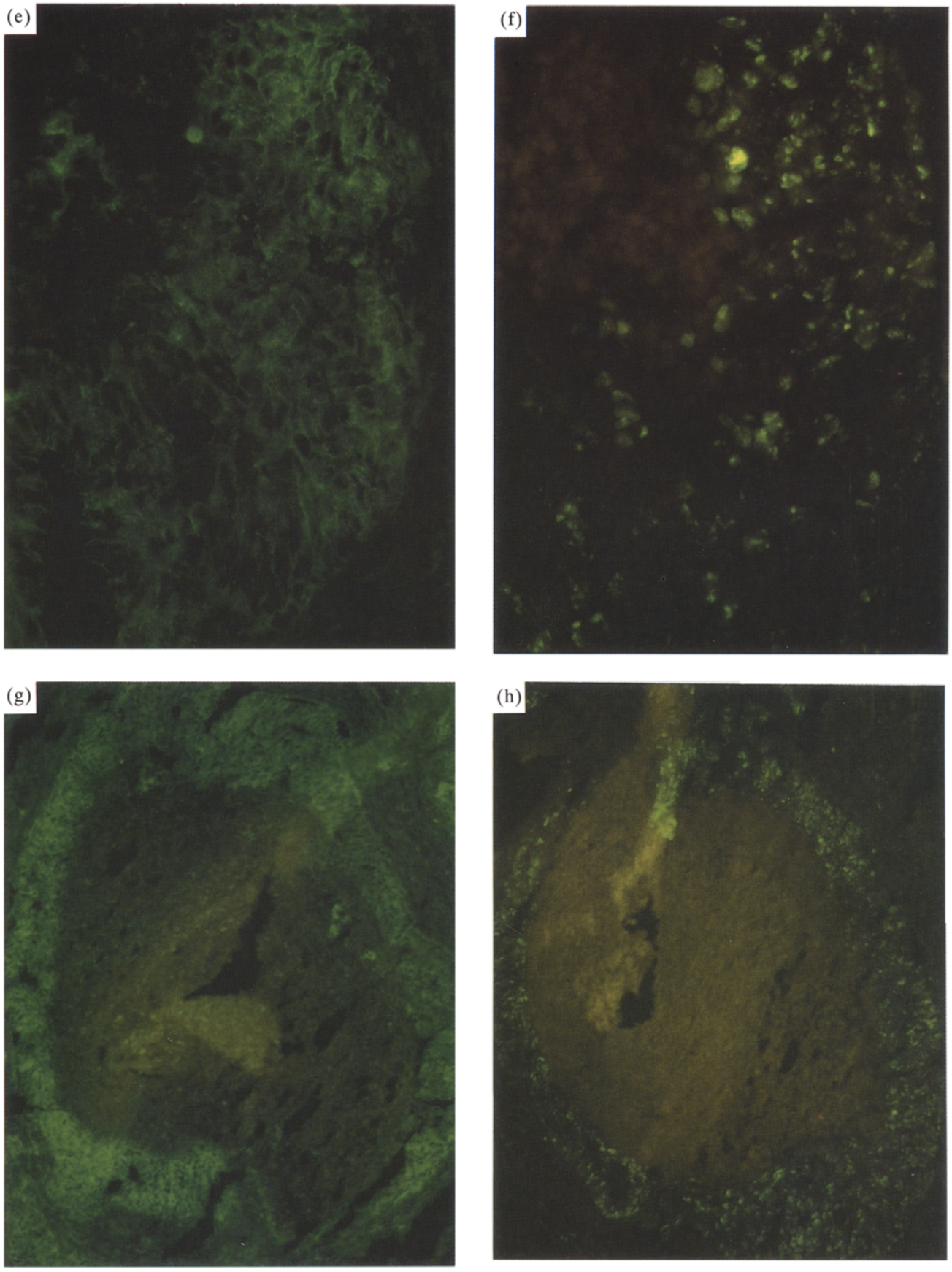


Fig. 1. (e)___ = 15μm. Well differentiated adenocarcinoma patient no. 4; CD44 staining is detected homogeneously in epithelial and stromal cells. (f = 15μm. Serial section to (e), staining for Ki-67 is positive in epithelial cells and negative on stromal cells. (g)___ = 60 μm. Moderately different adenocarcinoma, patient no. 9. CD44 staining detected in peripheral carcinomatous epithelial cells and some lymphoid cells in the surrounding str (h)___ = 60 μm. Staining for Ki-67 on serial section to (g) detects similar area to that stained with anti-CD44 in the carcinomatous cells but not i stromal cells. Both antibodies were negative in the necrotic area.

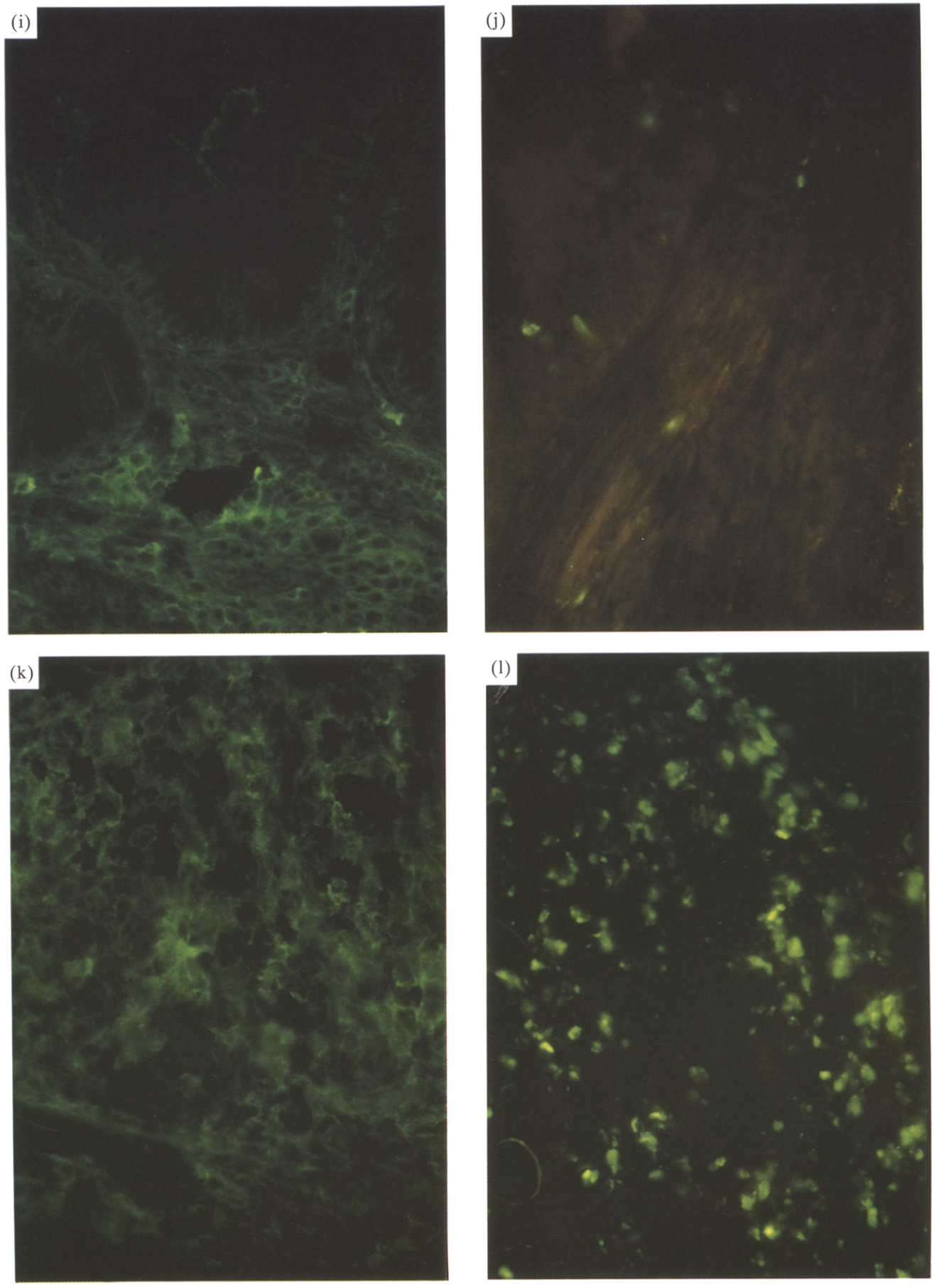
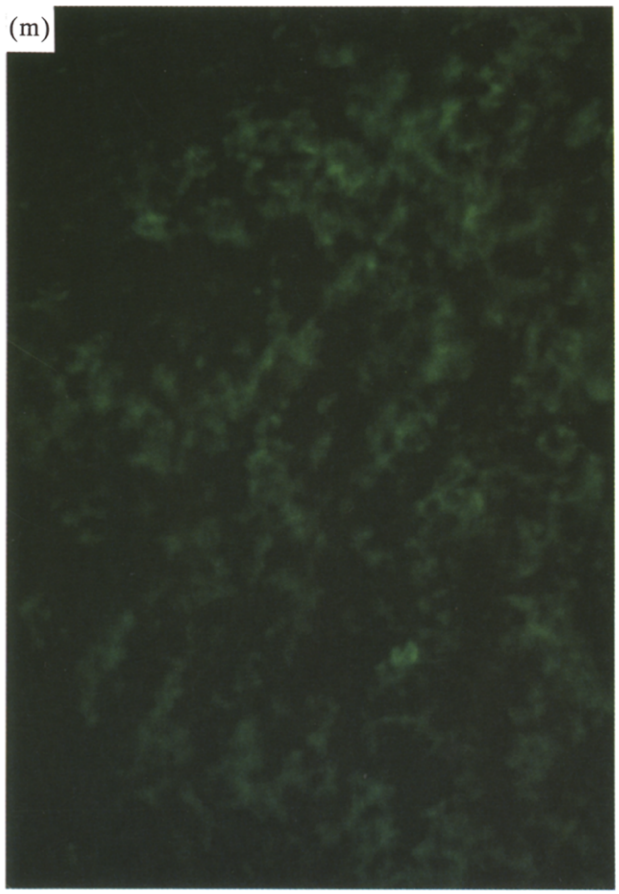


Fig. 1. (i)___ = 15μm. Moderately differentiated colonic adenocarcinoma, patient no. 16 showing strong CD44 staining of some lymphoid cells in the stroma but weak or negative staining in epithelial cells. (j)___ = 15μm. Staining for Ki-67 on serial section to (i) is almost negative in both epithelial cells and stromal cells. (k)___ = 15μm. Poorly differentiated adenocarcinoma patient no. 20; strong CD44 staining is detected homogeneously in epithelial cells. (l)___ = 15μm. Serial section to (k), nuclear staining for Ki-67 is positive.



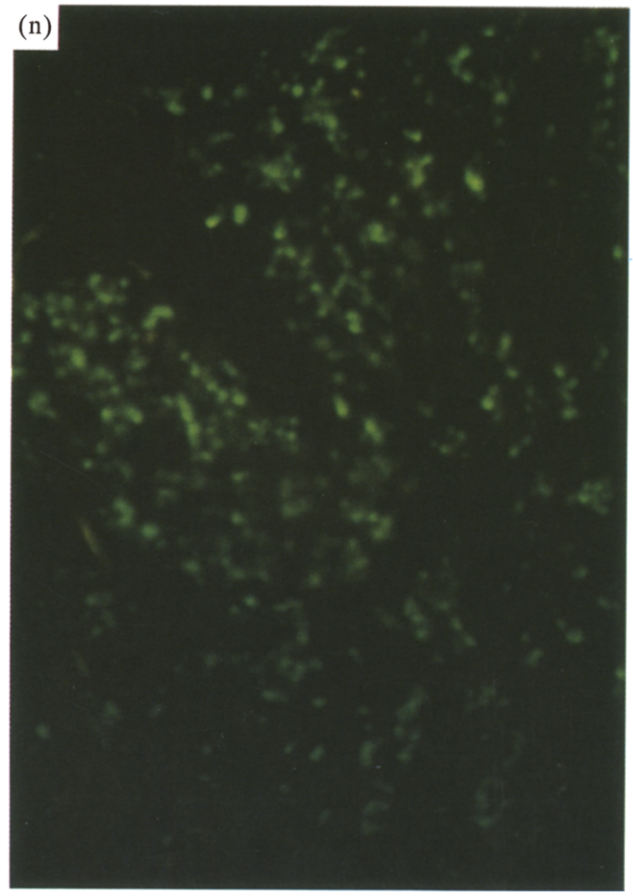


Fig. 1. (m)___ = 30μm. Metastatic adenocarcinoma (poorly differentiated) from patient no. 23; strong CD44 staining is detected homogeneously in tumour cells. (n)___ = 30μm. Serial section to (m), nuclear staining for Ki-67 is positive.

was heterogeneous in these samples and always detected in areas of tumour which stained positively with Ki-67. This is illustrated in Fig. 1m, which shows CD44 staining of a sample taken from patient 23 (Table 1) and Fig. 1n, which shows Ki-67 staining of a serial section.

DISCUSSION

The distribution of F10-44-2-reactive CD44 in the normal human colon and in progressive stages of colonic tumour development was investigated and compared with proliferative status defined with Ki-67. The results showed that CD44 expression was consistently associated with proliferation of epithelial cells. This was irrespective of tumour stage, grade or site; i.e. whether the sample was from a primary or metastatic origin. Non-proliferating areas of the carcinomatous epithelium did not express CD44, although non-proliferating lymphoid cells of the stroma did, which indicates the epithelial cell specificity of the CD44 proliferation association.

The association of CD44 with invasive human tumours and with metastases in animal models indicates a role for standard-type and variant CD44 in aiding tumour dissemination but our studies showed no apparent correlation of CD44 expression or cell proliferation with tumour progression, as there was no linear trend with Dukes' staging or differentiation (Table 1). Although the number of cases in our study was small, larger studies have also shown that proliferation status does not correlate with colorectal tumour stage or grade [25, 26].

In the three cases of hepatic metastases from colonic adenocarcinomas that were available in our study, CD44 showed a patchy distribution, similar to that seen in primary tumours; as for the primary tumours, CD44 expression was associated with areas of proliferation. This suggested that CD44 was primarily linked with the cells' proliferative activity rather than their metastatic potential. However, the areas of these tumours with significant CD44 expression may represent invasive and metastatic cells which were capable of redifferentiation (and loss of CD44 expression) once the metastasis had formed.

It is also important to note that the association of CD44 with metastases shown by other workers has been with CD44 variants [3, 4], as well as with standard-type [2] CD44. The variant forms would not be selectively detected with the F10-44-2 anti-CD44 used in our experiments as this antibody detects standard-type CD44. A number of tumour- and metastases-related CD44 gene products have recently been obtained by polymerase chain reaction (PCR) amplification across the splice site of CD44 from human breast and colon cancers [27]. Therefore, it should eventually be possible to obtain antibodies to these variants and to determine whether there are subsets associated with either metastases or proliferation.

In normal colonic mucosa CD44 staining was restricted to the lower third of the epithelial crypt, a result consistent with the location of the proliferative zone and with observations by other workers [12, 15]. Using the Ki-67 antibody we confirmed that these CD44 positive cells were in the process of cell division.

The association of CD44 with proliferation was always restricted to epithelial cells. Lymphoid cells in the lamina propria, and in the tumour stroma, often stained strongly with anti-CD44 but did not show corresponding staining with Ki-67 and, therefore,

did not appear to be dividing. This indicates CD44 had a separate function in these lymphoid cells, and supports observations that the CD44 cell adhesion molecule has distinct functions in different tissues [18].

The reason for association of CD44 with epithelial cell proliferation remains uncertain. Birch *et al.* found no correlation with CD44 expression and growth rate *in vitro*, although cells which were high CD44 expressors were more able to bind hyaluronic acid and to form metastases in hyaluronic acid-rich sites, than cells which were low CD44 expressors [2]. It is possible that the association of CD44 with proliferation is linked to hyaluronic acid binding. In the normal colon, CD44 is involved in cell substratum adhesion to hyaluronic acid, and provides the link between the cytoskeleton of colonic epithelial cells and the basement membrane [15, 28]. The absence of CD44 in mature cells at the tops of crypts could result in a loss of adhesiveness which must occur to allow the differentiated cells to slough off into the bowel lumen. This simple model allows an adhesion molecule role for CD44, and could indicate coincidental expression with proliferation. However, our results indicate a stronger association with proliferation since, in colonic tumours, CD44 was always present on proliferating cells irrespective of their differentiation status, tumour site or stage. Our work suggests that the F10-44-2-defined CD44/hyaluronic acid receptor may have an important role in supporting division in normal and cancerous colonic epithelial cells; since CD44 links with the cytoskeleton, this could be associated with cytoskeletal organisation in proliferating versus non-proliferating epithelial cells.

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