

ORIGINAL ARTICLE

Proliferating fibroblasts at the invading tumour edge of colorectal adenocarcinomas are associated with endogenous markers of hypoxia, acidity, and oxidative stress

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Background: Stroma frequently forms at sites of active tumour invasion, and may be important for tumour growth and progression. The term "stromatogenesis" is used to describe this unique process that involves host peritumorous fibroblasts and is very different to reactive fibrosis.

Aims/Methods: To investigate the activation status of host fibroblasts at the invading tumour edge, assessed as MIB1 proliferation index and thymidine phosphorylase (TP) expression. Results were related to vascular density and certain properties of invading cancer cells—MIB1 proliferation activity, TP expression, expression of endogenous markers of hypoxia (hypoxia inducible factor-1 α ; HIF1 α) and acidity (lactate dehydrogenase-5; LDH5). Standard immunohistochemical techniques were applied to 150 colorectal adenocarcinomas.

Results: Normal fibroblasts at the tumour edge had a median MIB1 index of 2%—significantly higher than normal submucosal fibroblasts (0.3%) and significantly lower than cancer cells (40%). Normal peritumorous fibroblasts with a proliferation rate above the median strongly expressed TP and were supported by an increased vascular network. Cancer cells close to these fibroblasts had a high MIB1 proliferative index, high HIF1 α and LDH5 reactivity, and a clear trend to extramural extension. All associations were significant.

Conclusions: These results suggest that activated fibroblastic status at the invading tumour front sets the stage for stromatogenesis and new blood vessel formation, facilitating deep transmural invasion in colorectal adenocarcinomas. This complicity of peritumorous fibroblasts in the overall aggressiveness/invasive and metastatic ability of colorectal tumours, occurring within the framework of cancer–stromal cell interactions, is probably favoured by the altered microenvironmental conditions of hypoxia and acidity.

The formation of new stroma at sites of active tumour cell invasion is a common event in human malignancy. We proposed the term "stromatogenesis" to denote the laying down of a specific stroma, after the proliferation of peritumorous fibroblasts and the disruption of normal tissue continuity facilitating tumour cell invasion.¹ It does not refer to usual fibrosis, which is generally thought to oppose tumour cell infiltration. This was acknowledged by the inspired work of many investigators who, simulating the conditions of invasion and metastasis, brought epithelial tumour cells into contact with normal fibroblasts in tissue cultures. In all cases, the presence of tumour associated, but not other, fibroblasts accelerated tumour growth. Thus, Fromigue *et al* reported a cascade of modulated genes encoding proteins involved in growth, angiogenesis, and invasion in normal lung fibroblasts, after coculture with tumour cells of primary non-small cell lung carcinomas.² Nakagawa *et al* found activation of approximately 170 of 22 000 genes in fibroblasts associated with metastatic colon cancer, including many genes encoding growth factors and cell adhesion molecules,³ whereas normal skin fibroblasts, not confronting tumour cells, remained inactive.

"The purpose of our study was to investigate the role of proliferating fibroblasts at the invading tumour front of colorectal adenocarcinomas in relation to tumour aggressiveness/invasiveness and metastatic ability"

It is probable that at the edge of the tumour important interactions occur between cancer cells, endothelial cells, and the tumour supporting stroma.⁴ The peritumorous fibroblasts, apparently stimulated by the invading tumour cells, may assume a synergic role in tumour growth, producing factors stimulating cancer cell proliferation and invasion. At the same time, cancer cells induce fibroblastic and endothelial cell activation, leading to stromatogenesis and new blood vessel formation.^{5–10} Yet, the most crucial questions remain: which factors stimulate fibroblastic proliferation? What is the precise role of stromatogenesis in tumour growth and invasion? What is the clinical relevance of such a process?

The purpose of our study was to investigate the role of proliferating fibroblasts at the invading tumour front of colorectal adenocarcinomas in relation to tumour aggressiveness/invasiveness and metastatic ability. Fibroblastic activation/proliferation is an important feature of stromatogenesis, and assessment of the MIB1 proliferation index in fibroblasts would provide a quantitative measure of the stromatogenic process. Variations in the rate of fibroblastic proliferation among tumours were also assessed and correlated with histopathological features, cancer metastatic ability, angiogenesis, and angiogenic factors (vascular endothelial growth factor; VEGF), endogenous markers of hypoxia (hypoxia

Abbreviations: HIF1 α , hypoxia inducible factor-1 α ; LDH5, lactate dehydrogenase-5; TP, thymidine phosphorylase; VD, vascular density; VEGF, vascular endothelial growth factor

Table 1 Details of the antibodies, dilutions, and antigen retrieval methods used

Primary antibody	Dilution/incubation time	Antigen retrieval	Specificity	Source
Ki-67	1/75 (75 min*)	MW†	MIB1	YLEM, Avezzano, Italy
P-GF.44C	1/4 (75 min*)	MW†	TP	Oxford University, Oxford, UK
VG1	1/4 (75 min*)	MW†	VEGF	Oxford University
JC70 (CD31)	1/20 (30 min*)	Protease‡	Endothelium	Dako, Glostrup, Denmark
ESEE 122	1/20 (overnight)	MW†	HIF1 α	Oxford University
9002	1/100 (75 min*)	MW†	LDH5	Abcam, Cambridge, UK

*At room temperature; †slides are placed in antigen unmasking buffer pH 6.0 (TAR001; YLEM) then microwaved (3×4 minutes); ‡predigestion with protease type XXIV for 20 minutes at 37°C.

HIF1 α , hypoxia inducible factor-1 α ; LDH5, lactate dehydrogenase-5; TP, thymidine phosphorylase; VEGF, vascular endothelial growth factor.

inducible factor-1 α ; HIF1 α), acidity (lactate dehydrogenase-5; LDH5), and cellular oxidative stress (thymidine phosphorylase; TP).

MATERIALS AND METHODS

The material was retrieved from the files of the department of pathology, Democritus University of Thrace Medical School, Alexandroupolis, Greece. Haematoxylin and eosin stained sections were reviewed and the most representative blocks of specimens exhibiting a clear invading tumour front, free of an inflammatory response, were selected. One hundred and fifty consecutive primary colorectal adenocarcinomas were selected. Approval had been obtained by the local research committee. The histological diagnosis, tumour differentiation, depth of local invasion, and lymph node metastasis were reaffirmed. In addition, 20 normal large intestinal samples were included as controls for assessing MIB1 proliferation activity in normal submucosal fibroblasts. Normal mucosal fibroblasts were not estimated in our study because of continuous replication and replacement of those lost through differentiation and subsequent desquamation. In 17 of 150 cases, metastasis to distant organs was present and surgery was performed mainly for palliative reasons. The tissues had been routinely fixed and processed.

Immunohistochemistry

Immunohistochemistry was performed to understand some facets of tumour–stroma interactions. To this end, sections were cut at 3 μ m and stained with the following immunohistochemical techniques: a standard streptavidin–biotin method for MIB-1, TP, VEGF, HIF1 α , and LDH5.^{11–15} Antigen retrieval was achieved by microwave heating or enzyme digestion. Antibody reactivity was detected with diaminobenzidine as chromogen. The slides were counterstained with Mayer's haematoxylin. The alkaline phosphatase–anti-alkaline phosphatase method and the JC70/anti-CD31 monoclonal antibody were used for endothelial cells to highlight blood vessels.¹⁶ Table 1 provides details of the primary antibodies used, the working dilutions, etc. Appropriate positive controls were included in each staining run. Non-specific sheep immunoglobulin IgG for LDH5 and mouse IgG for all other antibodies was substituted for the primary antibody as negative control, at a concentration where immunostaining of control slides gave a faint cytoplasmic staining.

Assessment of immunohistochemical results

Immunohistochemical assessment was performed independently by two observers (AG, ES) all along the invading tumour front. MIB1 stained sections were assessed using a ×40 objective and a semiquantitative method. The percentage of TP, VEGF, HIF1 α , and LDH positive cells was assessed using a ×20 objective. Discrepancies were resolved over a double headed microscope.

MIB1 is an antigen expressed in proliferating cells. The expression is nuclear. The proliferative activities of both

Table 2 HIF1 α and LDH5 grading system based on the intensity and extent of cytoplasmic and nuclear staining

HIF1 α /LDH5 grading system	Score
Complete absence of reactivity	Negative (low)
Weak cytoplasmic reactivity (regardless of extent)	Low
Strong cytoplasmic reactivity in less than 50% of tumour cells	Low
Nuclear expression in sporadic tumour cells (<10% of cells)	Low
Strong cytoplasmic expression in more than 50% of tumour cells	High
Nuclear expression in more than 10% of tumour cells	High

HIF1 α , hypoxia inducible factor-1 α ; LDH5, lactate dehydrogenase-5.

fibroblasts and tumour cells were evaluated. Ten fields were examined for each tumour. The MIB1 proliferating index was estimated as the percentage of fibroblasts or tumour cells with positive nuclei: positive fibroblasts/total fibroblasts and positive tumour cells/total tumour cells, respectively. The median value was used to divide colorectal tumours into groups of low and high MIB1 proliferation activity (index).

TP is an angiogenic factor and a marker of oxidative stress.¹⁷ TP expression is nuclear and cytoplasmic and was evaluated in both fibroblasts and tumour cells. TP reactivity in < 50% of fibroblasts or tumour cells was graded as low, whereas expression in more than 50% of cells was graded as high.

VEGF is another, probably more potent, angiogenic factor. VEGF was assessed in tumour cells. The expression is cytoplasmic. The percentage of VEGF positive cancer cells was assessed semiquantitatively using a ×20 objective. The median value was used as a cutoff point to define cases of high and low VEGF reactivity.

Angiogenesis was assessed in three areas of high vascular density (VD) at 20 ×10 magnification. The mean value of each section was recorded. The median VD was used as a cutoff point to define tumours of high and low angiogenic activity.

HIF1 α and LDH5 are endogenous markers of hypoxia and acidity, respectively. Expression is nuclear and cytoplasmic, and was estimated separately. The importance of assessing both cytoplasmic and nuclear staining for HIF expression has been raised in earlier studies.^{12–18–19} Although it is generally assumed that nuclear HIF is the active form, HIF protein is synthesised and degraded in the cytoplasm. It is possible that redistribution may occur during tissue collection, but this would be extremely difficult to be controlled; however, the overall expression indicates selective upregulation of the pathway in cancer. Analysis based on pure nuclear expression showed absence of or a very marginally significant association with other molecular factors or prognosis in several previous studies, indicating that it is the tumour specific

Table 3 Immunohistochemical results (reactivity range, median value, and grouping of cases)

Invading tumour front	Range	Median	High	Low
MIB1 index in fibroblasts	0–20%	2%	72	78
MIB1 index in cancer cells	2–90%	40%	64	86
TP expressing fibroblasts	0–100%	50%	80	70
TP expressing cancer cells	0–100%	0%	8	142
VEGF expressing cancer cells	0–90%	10%	44	106
Vascular density	15–110	42	74	76
HIF1 α expressing cancer cells nuc/cyt (grading scale)			58	92
HIF1 α nuc	0–30%	0%		
HIF1 α cyt	0–100%	5%		
LDH5 expressing cancer cells nuc/cyt (grading scale)			103	47
LDH5 nuc	0–70%	0%		
LDH5 cyt	0–100%	30%		

cyt, cytoplasmic staining; HIF1 α , hypoxia inducible factor-1 α ; LDH5, lactate dehydrogenase-5; nuc, nuclear staining; TP, thymidine phosphorylase; VEGF, vascular endothelial growth factor.

finding of strong cytoplasmic HIF expression that better reflects the HIF upregulated pathway in paraffin wax embedded material. This is in general accordance with the scoring system reported by Zhong *et al.*¹⁹ and formed the basis for a grading system (table 2) proposed and applied by our group in a series of previous studies.^{18 20–23} The same system was used in the current work to assign colorectal tumours into high and low HIF1 α and LDH5 reactivity groups, respectively.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 2.01 package (GraphPad, San Diego, California, USA). Fisher's exact test was used for testing relations between categorical variables as appropriate. A p value ≤ 0.05 was considered significant.

RESULTS

Table 3 shows the immunohistochemical results, as assessed all along the invading tumour front in 150 colorectal adenocarcinomas. There was excellent interobserver agreement in the evaluation of the MIB1 score ($r^2 = 0.94$; $p < 0.0001$).

Normal fibroblasts at the tumour edge showed a median MIB1 index of 2% (fig 1), significantly more than the 0.3% (range, 0.1–0.5%) growth fraction found in normal control submucosal fibroblasts. The median MIB1 proliferation index

in cancer cells at the tumour–stromal cell interphase was 40%.

TP reactivity in $> 50\%$ of cells was common in host peritumorous fibroblasts (fig 2), but not in cancer cells, where it occurred in only eight of 150 cases.

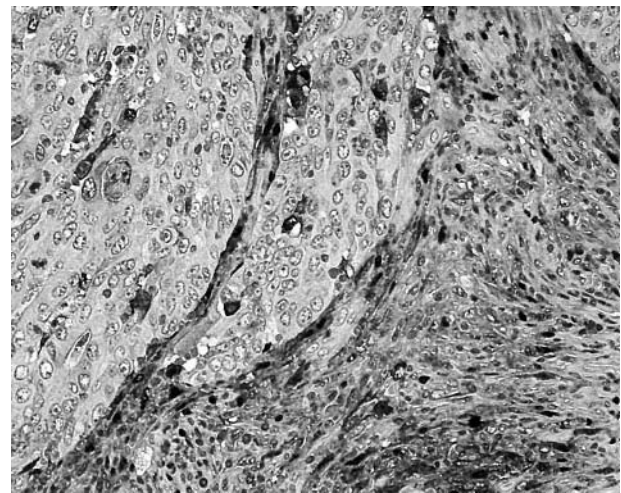


Figure 2 Fibroblasts at the invading tumour edge showing high nuclear/cytoplasmic thymidine phosphorylase expression.

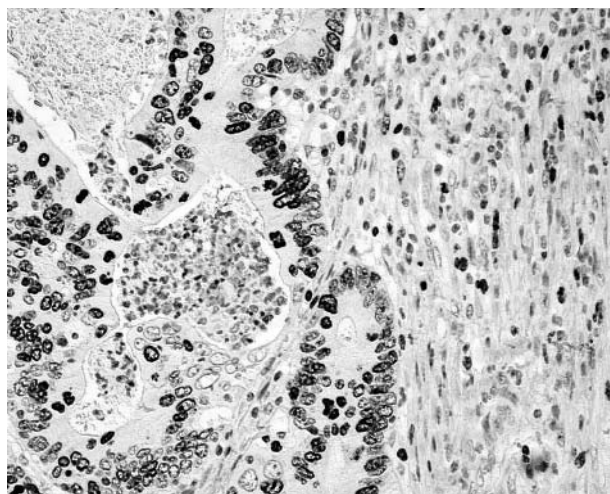


Figure 1 Fibroblasts at the invading tumour edge with high MIB1 proliferative activity.

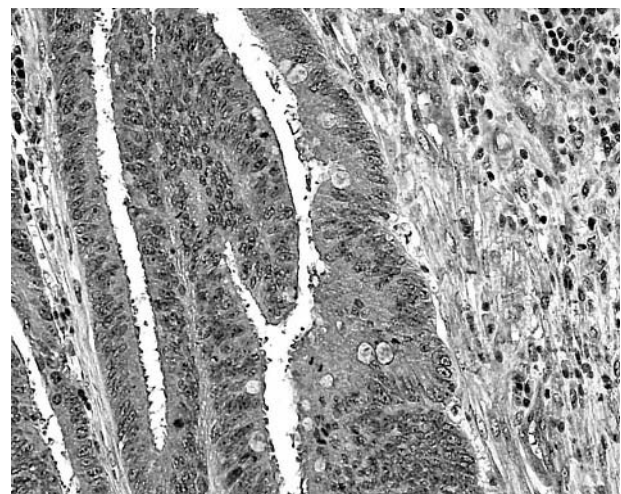


Figure 3 Cytoplasmic and nuclear expression of hypoxia inducible factor-1 α in cancer cells at the invading tumour edge.

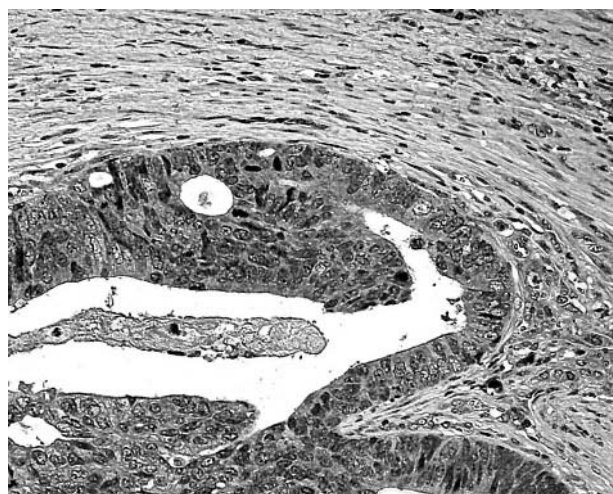


Figure 4 Cytoplasmic expression of vascular endothelial growth factor in cancer cells at the invading tumour edge.

HIF1 α reactivity (fig 3) was seen in a median of 5% of cells, and for LDH5 this figure was 30%.

Between 0% and 90% of cancer cells expressed VEGF (fig 4), with a median value of 10%.

The VD ranged from 15–110, with a median value of 42.

MIB1 fibroblastic activity in association with other parameters

Fibroblasts with a high MIB1 proliferation index at the invading tumour edge were associated with high TP reactivity and increased VD at this site (table 4). There was a significant correlation between high fibroblastic growth fraction, as assessed by MIB1 immunostaining, and high MIB1 index in cancer cells. A positive correlation was also established between MIB1 fibroblastic proliferation and deep transmural invasion with extension to the pericolic fat. No association was noted between MIB1 index and tumour dimension or anatomical location (table 5).

Furthermore, peritumorous fibroblasts with a high MIB1 index most often expressed the endogenous markers of hypoxia (HIF1 α) and intratumoral acidity (LDH5) (table 6). Their association with the angiogenic protein VEGF was only marginal.

There was no association between MIB1 proliferation index in peritumorous fibroblasts and lymph node or distant metastasis.

DISCUSSION

Stromatogenesis is the formation of new stroma occurring, in parallel with the neoplastic process, at sites of active tumour invasion—that is, at the free surface of a developing

Table 4 MIB1 proliferation index in fibroblasts at the invading tumour front and its association with stromal angiogenesis

	MIB1 proliferation index in fibroblasts		
	Low	High	p Value
TP in fibroblasts			
Low	46	24	0.001
High	32	48	
VD			
Low	46	30	0.04
High	32	42	

TP, thymidine phosphorylase; VD, vascular density.

Table 5 MIB1 proliferation index in fibroblasts at the invading tumour front and its association with primary tumour characteristics

	MIB1 proliferation index in fibroblasts		
	Low	High	p Value
MIB1 cancer cells			
Low	60	26	<0.0001
High	18	46	
Tumour dimensions			
≤ 4 cm	32	29	0.49
> 4 cm	36	43	
Location			
Rectum	22	18	0.65
Sigmoid	20	16	
Colon	36	38	
Transmural invasion			
No	36	22	0.05
Yes	42	50	

Table 6 MIB1 proliferation index in fibroblast at the invading tumour front and its association with parameters indicative of hypoxic stress and acidity

	MIB1 proliferation index in fibroblasts		
	Low	High	p Value
VEGF			
Low	60	46	0.07
High	18	26	
HIF1 α			
Low	56	36	0.006
High	22	36	
LDH5			
Low	30	17	0.05
High	48	55	

HIF1 α , hypoxia inducible factor-1 α ; LDH, lactate dehydrogenase; VEGF, vascular endothelial growth factor.

exophytic tumour, at the invading tumour front of an advancing endophytic tumour, and at sites of tumour metastasis, wherein the newly formed stroma disrupts the continuity of normal structures, cleaving pathways for the invading tumour cells. Stromatogenesis is of utmost importance for tumour growth and progression, with the new stroma sustaining the invading tumour cells and giving grounds for angiogenesis. The newly formed tumour associated spindle cells are qualitatively altered, having an activated gene profile compared with normal non-activated fibroblasts^{2–3} and also (as was shown immunohistochemically in our study) expressing α smooth muscle actin, but not vimentin or desmin (data not shown), suggesting a shift to myofibroblastic differentiation. This is in accordance with a recent report of ours.¹

Stromatogenesis occurs in almost all types of invading tumours, and some form of quantitation would be useful in assessing tumour aggressiveness. Perhaps the enumeration of spindle cells in a substratum harbouring tumour cells (rate of fibroblast proliferation) may prove effective. In our study, there was increased MIB1 proliferative activity in the host fibroblasts at the invading tumour edge compared with the very low, almost negligible, proliferation of fibroblasts in normal colonic submucosa. The reason for this accelerated proliferative activity should be sought in tumour–stroma interactions. For example, the secretion of fibronectin, vitronectin, and other soluble factors from colonic cancer cells induces new stroma formation through activation, migration, and proliferation of host fibroblasts.^{24–25} The loss

of responsiveness to transforming growth factor β in mouse fibroblasts results in the development of invasive carcinoma in parallel with the production of abundant stroma.²⁶

We also found that the increased proliferation rate in the peritumorous fibroblasts was accompanied by an analogous, or rather exaggerated, increase in MIB1 activity (index) in cancer cells. This is in accordance with kinetic studies, exploiting coculture models of cancer cells and normal tumour confronting fibroblasts, but not other normal fibroblasts. In such cultures, Hauptmann *et al* demonstrated proliferative activity in both colonic fibroblasts and colorectal cancer cells.²⁷ Camps *et al* succeeded in accelerating growth in human epithelial tumours after coinoculation of cancer cells with transformed fibroblasts in athymic mice.²⁸ Olumi *et al* stimulated tumour growth and progression in initiated prostate epithelial cells after they were cocultured with tumour associated prostatic fibroblasts.²⁹ Shekhar *et al* indicated that tumour affiliated breast fibroblasts induce the proliferation of cancer cells and ductal alveolar morphogenesis in cocultures with breast cancer cells.³⁰ This was further augmented in the presence of active angiogenesis.³⁰

"The altered physiological conditions of hypoxia and acidity may contribute to the activation of peritumorous fibroblasts, enhancing further tumour aggressiveness"

In agreement, we found that the highly activated status of the tumour confronting fibroblasts and the fibroblast confronting tumour cells, assessed by MIB1 proliferation index along the invading tumour front, was associated with deep transmural cancer cell invasion in the presence of TP and increased angiogenesis. TP overexpression is an endogenous marker of oxidative stress, promoting thymidine catabolism to 2-deoxy-D-ribose-1-phosphate, a strongly reducing sugar that generates radical oxygen species during protein glycation.^{17–31} Under such stress conditions, tumour cells secrete the angiogenic factors VEGF and interleukin 8, together with matrix metalloproteinases.¹⁷ This would explain why TP expressing fibroblasts may promote angiogenesis, invasion, and metastasis, as was confirmed in several clinicopathological studies.^{23–32–34} Along these lines, it was also shown that cell–cell contact between cancer cells and tumour supporting fibroblasts enhanced the production and activation of matrix metalloproteinases by cancer cells, promoting pericellular proteolysis, angiogenesis, and tumour cell invasion.^{35–36} Similarly, in the previously cited study of Shekhar *et al*,³⁰ the inductive morphogenic effects of tumour associated fibroblasts was further augmented by the inclusion of endothelial cells with a dramatic increase in proliferation of duct and acini, which was accompanied by increased expression of matrix metalloproteinases, degradation of the extracellular matrix, and, finally, tumour cell invasion.³⁰ Others have suggested that the stimulatory effect of peritumorous fibroblasts on cancer cell migration is probably mediated through the secretion of a "migration stimulating factor", which has been effectively shown in skin fibroblasts from patients with breast cancer,³⁷ or through the expression of integrin chains in tumour cells to promote fibronectin mediated tumour cell invasion.³⁸ Although an association between increased proliferation of host fibroblasts at the invading tumour edge and metastasis either to lymph nodes or distant organs was not confirmed in our present study, this does not preclude a complementary role for activated fibroblasts in the metastatic process, perhaps through an individual cancer cell genotype or activated angiogenesis.

Another, equally, important observation of our study was the direct association between increased fibroblastic proliferation and overexpression of HIF1 α and its downstream

Take home messages

- In colorectal adenocarcinomas, the high proliferation index and expression of thymidine phosphorylase in host fibroblasts at the invading tumour edge suggest that the activation of fibroblasts at this site sets the stage for stromatogenesis and new blood vessel formation, facilitating deep transmural invasion
- The involvement of peritumorous fibroblasts in the overall aggressiveness/invasive and metastatic ability of colorectal tumours, via cancer–stromal cell interactions, is probably favoured by the altered microenvironmental conditions of hypoxia and acidity

gene LDH5. Given that HIF1 α induction is hypoxia dependent,³⁹ such tumours should be hypoxic and, presumably, acidic, mainly because of the biochemical transformation of pyruvate to lactate suggested by the high expression of LDH5.⁴⁰ The ability of fibroblasts to proliferate under hypoxic conditions has been confirmed experimentally,⁴¹ and may have an important role in the development of pulmonary hypertension, a condition where hypoxia induces proliferation of adventitial fibroblasts in the pulmonary artery.^{42–43} This is probably because hypoxia increases ATP concentrations in adventitial fibroblasts and endothelial cells, promoting DNA synthesis and proliferation.⁴⁴ Anderson *et al* showed in normal rat fibroblasts a three stage response to anoxia, consisting of: (a) transcription, (b) induction of several intracellular proteins, and (c) secretion of three major proteins, including the protease cathepsin L.⁴⁵ What is most interesting is that in the authors' opinion fibroblasts, under anoxic conditions, show several characteristics of malignant cells. The response of fibroblasts to acidic conditions is not well understood. A recent study has reported that lactate increases intracellular oxidants, promoting proliferation of cultured fibroblasts in a dose dependent manner.⁴⁶ Another study showed that platelet induced proliferation of fibroblasts was enhanced at an acidic pH, suggesting an interplay of platelet aggregation and tumour acidity in the induction of fibroblastic growth.⁴⁷

We conclude that host fibroblasts at the invading tumour edge have a high activation status, as suggested by the high proliferation activity and the overexpression of TP. The intensity of this fibroblastic activity is related to stromatogenesis, increased blood vessel formation, and invasion of colorectal cancer cells through the muscle wall into the serosa and the pericolic fat. The close association of proliferative activity of host fibroblasts at the invading tumour edge with endogenous markers of hypoxia and acidity suggest that, apart from the stroma–cancer interactions, the altered physiological conditions of hypoxia and acidity may contribute to the activation of peritumorous fibroblasts, enhancing further tumour aggressiveness.

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