Stromal tenasin distribution as a prognostic marker in colorectal cancer

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Summary A total of 169 colorectal adenocarcinomas, obtained from patients with a median follow-up of 6.5 years, were studied with immunohistochemical staining on cryosections using a monoclonal anti-tenasin antibody to evaluate the possible association between the staining patterns and tumour stage, tumour differentiation and survival. We found two different staining patterns in the tumour stroma—a diffuse stromal fibrillar staining in 92 out of 169 (54%) tumours and a subglandular staining in the remaining 77 tumours. When the entire group of patients (P < 0.01) and the group of potentially cured patients (P < 0.03) were analysed univariately, it was found that diffuse stromal fibrillar staining was associated with a shorter survival time than subglandular staining. In a multivariate analysis, the Dukes' stage and age were independent prognostic factors, whereas the tenasin expression did not retain a clear independent relationship to survival (P = 0.06). Hence, it appears that the tumour expression of tenasin may be a potential prognostic marker in colorectal cancer, in so far as a diffuse stromal fibrillar staining pattern seems to indicate an increased risk of poor outcome. However, after adjustment for age and Dukes' stage, the additional prognostic value of tenasin remains to be established in further analyses.

Keywords: tenasin; colorectal cancer; tumour stage; tumour differentiation; survival; immunohistochemistry

Tenasin is a large hexamic extracellular matrix protein that is present during embryonic development but essentially absent in normal adult tissues (Schenk and Chiquet-Ehrismann, 1994) and is expressed only at low levels or in a very restricted distribution. Several tenasin isoforms have been described (Siri et al, 1995). Tenasin has been suggested to be of importance in the normal healing process and in tumours, in which its role is presumed to be connected with cell adhesion and detachment, cell growth, cell migration and angiogenesis (Mackie et al, 1988; Eckblom and Auwerheide, 1989; Erickson and Bourdon, 1989; Chiquet-Ehrismann, 1993; Hahn et al, 1995; Joshi et al, 1995). It has been proposed that, although tenasin has no cell adhesion activity, it does affect the cell shape and, thus, may inhibit cell attachment to other extracellular proteins, including fibronectin (Mackie, 1994). It is also thought that tenasin plays a role in coordinating the provisional extracellular matrix surrounding the cancer tissue (Sakakura and Kusakabe, 1994).

Several reports have shown an up-regulation of tenasin in various tumour stroma, such as breast (Moch et al, 1993; Shoji et al, 1993; Yoshida et al, 1995), lung (Natali and Zardi, 1989), prostatic (Ibrahim et al, 1993) and gastric carcinomas (Ikeda et al, 1995; Illunga and Iriyama, 1995). Studies of this putative marker of the tumour matrix are also of considerable interest in colorectal cancer (Sugawara et al, 1991; Riedel et al, 1992; Sakai et al, 1993; Hauptmann et al, 1995; Riedel et al, 1995) because of the potential involvement of tenasin (or tenasin-like proteins) in cell adhesion and invasion during the metastatic process. Moreover, diagnostic and possibly therapeutic monoclonal antibodies, specific for the larger tenasin isoforms present in tumours, have been developed (Leprini et al, 1994).

In order to search for prognostic markers in colorectal cancer, especially in Dukes' stages B and C, which are associated with a high relapse rate (and for which adjuvant therapy could be an option), we investigated the expression of tenasin in 169 tumours from a prospective series of patients resected for colorectal cancer. The staining patterns were evaluated in relation to common clinicopathological variables and patient survival time.

MATERIALS AND METHODS

Patients

Tumour samples were collected from 169 consecutive patients operated on for colorectal cancer between January 1987 and November 1992. There were 72 men and 97 women of ages ranging from 40 to 92 (median 71) years. One hundred and forty-seven tumours (87%) were resected for cure: 36 in Dukes' stage A, 79 in stage B and 32 in stage C. The remaining 22 patients had perioperatively detected distant metastases and underwent a palliative resection. Tumour differentiation was characterized as good in 22 tumours, moderate in 110 and poor in 37. At follow-up in October 1995, 65 (39%) patients had died from other causes, without any indication of tumour relapse. Median survival time of living patients was 83 months (range 39-105 months).

Tumour biopsies

Tumour biopsies were snap frozen in dry-ice isopentane and stored at -70°C. Routine biopsies were taken for histopathological
Immunohistochemical detection

Acetone-fixed 6-μm cryosections were stained with the mouse monoclonal antibody BC-4 to tenascin (kindly provided by Dr P Ekblo, Department of Zoophysiology, Biomedical Center, University of Uppsala, Sweden). The avidin–biotin method, containing a peroxidase conjugate, was used (ABC Elite, Vector, Burlingame, CA, USA). The primary antibody was used at a dilution of 1:125 in phosphate-buffered saline supplemented with 5% normal horse serum and 1% bovine serum albumin, and was incubated with tissues for 60 min at room temperature. The primary antibody was then omitted and was replaced by either dilution buffer or normal mouse IgG, as negative controls. The secondary antibody, biotinylated horse anti-mouse IgG from Vector Laboratories, was used at a dilution of 1:200 and was incubated for 30 min.

Immunohistopathological evaluation

Tenascin staining patterns were examined by light microscopy, at a magnification of ×125. Coded slides from all 169 tumours were evaluated without prior knowledge of tumour stage or clinical outcome. In order to assess the interobserver agreement, two of the authors (UK, GL) evaluated 20 randomly selected and blinded sections.

Statistical methods

The Cox proportional hazards model was used (Lawless, 1992) in both the univariate and the multivariate survival analyses. Likelihood ratio and Wald tests were used in the testing. Survival curves were constructed using the Kaplan–Meier method, and differences between curves were tested using the log-rank test. The proportion of patients with diffuse tenascin expression among different categories of Dukes’ stage and differentiation was analysed using the logistic regression model. Trend tests were also used for these ordinarily scaled variables to increase the statistical power.

RESULTS

Tenascin staining patterns

Extensive fibrillar tenascin positivity was constantly seen in the tumour matrix, whereas tumour epithelial cells were entirely negative. Tenascin expression was generally seen in muscularis mucosae, in muscularis propria and in the vessel walls. Minimal staining, if any, was detectable in the adjacent normal bowel wall.

Table 1 Tenascin expression in colorectal cancer and its relation to tumour stage and tumour differentiation

|                  | Cases n | Tenascin diffuse staining n (%) | Death in cancer (%) | Tenascin subglandular staining n | Death in cancer (%) |
|------------------|---------|--------------------------------|---------------------|---------------------------------|---------------------|
| **Dukes’ stage** |         |                                |                     |                                 |                     |
| A                | 36      | 18 (50)                         | 4 (22)              | 18                              | 1 (6)               |
| B                | 79      | 38 (48)                         | 15 (39)             | 41                              | 6 (15)              |
| C                | 32      | 21 (65)                         | 11 (52)             | 11                              | 6 (55)              |
| D                | 22      | 15 (68)                         | 15 (100)            | 7                               | 7 (100)             |
| **Tumour differentiation** | |                                |                     |                                 |                     |
| Good             | 22      | 6 (27)                          | 2 (33)              | 16                              | 2 (12)              |
| Moderate         | 110     | 62 (56)                         | 25 (40)             | 48                              | 14 (29)             |
| Poor             | 37      | 24 (65)                         | 18 (73)             | 13                              | 4 (31)              |
There were two different tenasin stromal fibrillar staining patterns—a subglandular pattern, in which the tenasin staining outlined the border of the malignant tubules, and a diffuse pattern, characterized by diffuse interglandular stromal fibrillar distribution (Figure 1A and B). Either staining pattern was invariably seen throughout the interglandular stroma in each section. We did not see any correlation between tenasin expression and the number of microvessels in the tumour sections. No interobserver disagreement was seen.

**Tenasin stainings and tumour stage, tumour differentiation and survival time**

Dukes' stages C and D showed a tendency to have an increased proportion of diffuse tenasin staining. Compared with the reference category, Dukes' stage A, the increase was not significant (P-values 0.20 and 0.18), but a trend test yielded a result that was almost statistically significant (P = 0.06). There also appeared to be a connection between tumour differentiation and tenasin staining pattern, with a higher proportion of diffuse pattern among patients with moderately and poorly differentiated tumours (P-values 0.02 and 0.01 respectively; Table 1). The table also shows that the number of patients who died from cancer, or from other causes but with cancer, varied according to the Dukes' stage, tumour differentiation and tenasin staining pattern.

**Table 2** Univariate analyses showing the effects of age, tenasin expression, tumour differentiation and Dukes' stages on survival in patients resected for colorectal cancer

| Variable                  | β   | s.e. (β) | P-value | RH |
|---------------------------|-----|----------|---------|----|
| Age (continuous)          | 0.02| 0.01     | NS      | 1.02|
| Tenasin expression        |     |          |         |    |
| Subglandular              | 0.00| 0.27     | 0.007   | 2.07|
| Diffuse                   | 0.73| 0.27     |         |    |
| Tumour differentiation    |     |          |         |    |
| Good                      | 0.00| 0.41     | NS      | 1.31|
| Moderate                  | 0.27| 0.41     |         |    |
| Poor                      | 1.14| 0.44     | 0.01    | 3.11|
| Dukes' stage              |     |          |         |    |
| A                         | 0.00| 0.49     | 0.0001  | 33.68|
| B                         | 0.59| 0.47     | NS      | 1.80|
| C                         | 1.51| 0.47     | 0.001   | 4.53|
| D                         | 3.51| 0.49     | 0.0001  | 39.90|

RH, relative hazard. Number of patients, 169; number of deaths, 65.

**Table 3** Multivariate analysis showing the effects of age, tenasin expression, tumour differentiation and Dukes' stages on survival in patients resected for colorectal cancer

| Variable                  | β   | s.e. (β) | P-value | RH |
|---------------------------|-----|----------|---------|----|
| Age (continuous)          | 0.039| 0.014   | 0.005   | 1.04|
| Tenasin expression        |     |          |         |    |
| Subglandular              | 0.00| 0.28     | 0.056   | 1.72|
| Diffuse                   | 0.54| 0.28     |         |    |
| Tumour differentiation    |     |          |         |    |
| Good                      | 0.00| 0.42     | NS      | 1.47|
| Moderate                  | 0.38| 0.42     |         |    |
| Poor                      | 0.49| 0.47     | NS      | 1.63|
| Dukes' stage              |     |          |         |    |
| A                         | 0.00| 0.49     | 0.0001  | 39.90|
| B                         | 0.63| 0.47     | NS      | 1.86|
| C                         | 1.35| 0.48     | 0.005   | 3.90|
| D                         | 3.69| 0.52     | 0.0001  | 39.90|

RH, relative hazard. Number of patients, 169; number of deaths, 65.

Altogether, 39 (51%) of the 77 patients with tumours displaying a subglandular staining pattern were alive after 5 years, while the corresponding figure for the 92 patients with diffuse staining was 38 (41%). The relation between the type of tenasin expression and the cancer-specific survival time, when analysed with a life-table technique, showed a significantly shorter survival time for those patients whose tumours showed a diffuse pattern than for those who showed a subglandular pattern. This was valid for both the entire group of patients (log-rank, P < 0.01, Figure 2A) and the subset of patients with tumours in Dukes' stage A-C (log-rank, P < 0.03, Figure 2B). Univariate survival analyses showed a significant relationship to survival for Dukes' stage, tumour differentiation and diffuse tenasin staining pattern (Table 2). In a multivariate analysis encompassing all patients, Dukes' stage and age were independent prognostic factors for survival, whereas the diffuse tenasin staining pattern showed a borderline (P = 0.056) significant relationship to the survival time (Table 3). The relative
hazard (RH) for patients with diffuse expression was 1.72 (95% confidence limits 0.98–2.99). Tumour differentiation lost its relationship to survival.

**DISCUSSION**

All specimens investigated exhibited strong tenascin staining of the interglandular tumour stroma, whereas they predominantly lacked tenascin staining in the adjacent normal bowel wall, thus indicating tenasin up-regulation in malignancy. This finding has been described by others in colorectal cancer (Sugawara et al, 1991; Riedel et al, 1992, 1995; Sakai et al, 1993; Hauptmann et al, 1995) and in several other tumour types (Natali and Zardi, 1989; Ibrahim et al, 1993; Moch et al, 1993; Shoji et al, 1993; Ikeda et al, 1995; Ilunga and Iriyama, 1995; Yoshida et al, 1995). Like Sugawara et al (1991), we also found two different staining patterns. In contrast, Riedel et al (1992) did not discriminate various tenasin staining patterns but reported various degrees of extensive positivity in the basement membranes of colorectal carcinomas. Interestingly, Riedel et al (1995) reported that high serum levels of tenasin reflected metastatic disease.

Consistent with the findings of Sugawara et al (1991), we were able to demonstrate distribution differences between the two tenasin staining patterns according to Dukes’ stage and tumour differentiation. In addition, we established a significantly shorter survival time for patients showing the diffuse tenasin staining pattern than for patients showing the subglandular tenasin staining pattern. A possible explanation for this difference in prognosis between the two staining patterns could be that subglandular tenasin may fulfill a protective function in preventing tumour invasion and/or metastases, as suggested previously (Sakakura and Kusakabe, 1994; Siri et al, 1995).

It is recognized that until now the Dukes’ classification has been the best known prognostic factor in colorectal cancer (Lindmark et al, 1994; Bosman, 1995). However, there is also considerable variation in prognosis within the Dukes’ stages (Newland et al, 1987; Lindmark et al, 1994). Bentzen et al (1987) showed, for example, that some subgroups of Dukes’ stage C patients had better prognosis than some subgroups of Dukes’ stage B patients. For therapeutical and surveillance reasons, it is of importance to find stronger prognostic factors other than the original Dukes’ classification, i.e., to be able to predict those patients with tumours in Dukes’ stages B and C who are likely to have micrometastases requiring additional immediate treatment. Strong efforts have been made in the search for such markers that can replace or add information to that of the Dukes’ stage. Jass et al (1987) have reported a prognostic scoring system that was found to be superior to that of the Dukes’ stage for rectal cancer. The system considered the character of the invasive margin, peritumoral lymphocytic infiltration, local spread and number of metastatic lymph nodes. However, this scoring classification system has not been established, as yet, in clinical pathological practice, probably because the pathological examination is rather time-consuming. Several tumour markers also provide additional prognostic information, but their clinical relevance is yet to be defined as they mainly identify patients who already have metastases at diagnosis (Stähle et al, 1988; Lindmark et al, 1995). We have recently observed that the number of microvessels in tumour sections contributes to the prognosis prediction in colorectal cancer (Lindmark et al, 1996). However, in the present study, we did not see any correlation between the tenasin expression and the number of microvessels in the tumour sections. Tumour cell DNA ploidy and cell proliferation, measured by flow cytometry, have not turned out to be strong prognostic factors beyond that of Dukes’ stage in colorectal cancer (Lindmark et al, 1991; Botterg et al, 1993). This also appears to be the case for p53 overexpression, although some studies have shown a correlation with prognosis (Mulder et al, 1995; Kressner et al, 1996). Recent molecular studies suggest that the process of tumorigenesis in colorectal cancer proceeds through a series of genetic alterations (Fearon and Vogelstein, 1990). Two reports have shown that allellic loss on chromosome 18q (DCC gene) is associated with poor prognosis in Dukes’ stage B (O’Connel et al, 1992; Jen et al, 1994). Further studies are needed, however, to clarify how genetic alterations can contribute to prognosis. As yet, no single studied prognostic marker has proved to be better for prediction than the Dukes’ stage.

To summarize, the stromal fibrillar distribution of tenasin appears to be a potential prognostic marker. We were able to discriminate, on the basis of significant tenasin staining differences, between living patients with no evidence of disease and those who had died from cancer after a potentially curative resection for colorectal cancer. However, the strength of this finding was reduced after adjustment for the effects of Dukes’ stage and age.

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