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Differential expression of transforming growth factors-β1, -β2 and -β3 in human colon carcinoma

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Abstract

Transforming growth factor (TGF)-β is a protein family which affects multiple cellular functions including survival, proliferation, differentiation and adhesion. Among the three known isoforms, TGF-β1 is commonly overexpressed in solid malignancies. Recent studies in knock-out mice demonstrated non-redundant roles of different TGF-β isoforms in development. The present study was performed to assess tumour-associated expression of the three TGF-β isoforms in colon carcinoma. We report that colon carcinoma progression is associated with gradual and significant increases in expression of TGF-β1 and TGF-β2 mRNA and proteins. By contrast, TGF-β3 expression was detected in normal colonic mucosa and, at slightly higher levels, in tumour tissues. In addition, plasma levels of both TGF-β1 and TGF-β2 were significantly higher in cancer patients when compared with unaffected individuals. Taken together, our results indicate distinct expression patterns of the three TGF-β isoforms in colon carcinoma cells and possible systemic effects of TGF-β1 and TGF-β2 in tumour patients.

Keywords: Transforming growth factor-β; Colon carcinoma; Tumour progression

1. Introduction

Inappropriate expression of growth factor and cytokine genes frequently occurs during neoplastic development. In many cases, tumour-derived cytokines have been shown to aid survival or growth of the tumour cells themselves (autocrine effects) or to induce normal cells in the tumour bed to provide support for tumour development (paracrine effects). Because of their capacity to support tumour growth and development, tumour-derived cytokines are being considered as targets for therapeutic intervention in cancer. To guide these efforts, the expression patterns and relative contribution of individual tumour-derived cytokines to overall tumour development needs to be defined as precisely as possible.

Among tumour-derived cytokines, transforming growth factor (TGF)-β occupies an enigmatic position. TGF-β belongs to a large family of related factors that affect multiple processes including cell cycle progression, cell survival and differentiation during prenatal development and in the adult organism (for review, see [1]). The superfamily of TGF-βs includes over 30 members that are divided into four groups: the TGF-βs themselves, bone morphogenetic proteins, activins, and growth/differentiation factors. The mammalian TGF-β subfamily consists of three members with similar structure and function, i.e. TGF-β1, TGF-β2, and TGF-β3. *In vitro*, these TGF-β isoforms induce cell cycle arrest in normal and some malignant epithelial cells by inducing inhibitors of cyclin-dependent kinases [2–6]. Of note, malignant epithelial cells, including those derived from the gastrointestinal tract, frequently have acquired resistance to the growth inhibitory effects of TGF-β. TGF-β resistance may be accomplished by inactivating mutations [7–11] or downregulation [12] of TGF-β

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receptors or deletion or mutation of elements of TGF-β signal transduction pathways [13,14]. A wide variety of human tumours including many epithelial cancers overexpress TGF-β1 *in vitro* and *in situ*. This has also been observed in colorectal carcinomas, which express high levels of TGF-β1 in tumour tissue associated with elevated TGF-β1 serum levels [15–17].

In the present study, we examined expression patterns for all three TGF-β isoforms in colorectal carcinoma tissue and determined serum levels of TGF-β1 and TGF-β2 in colon carcinoma patients. This investigation was prompted by recent findings that different TGF-β isoforms perform non-overlapping functions, at least in embryonal development [18]. We describe distinct expression patterns of TGF-β1, TGF-β2 and TGF-β3 mRNA and proteins at different stages of colon carcinoma progression *in situ*. In addition, we provide evidence for high serum levels of not only TGF-β1 but also TGF-β2 in sera obtained from colon carcinoma patients.

2. Patients and methods

2.1. Tissue samples and patients

39 colon carcinoma patients (21 men and 18 women, aged 34–86 years, mean age 69 years) who underwent surgical resections at the Medical-Surgical Department, Molinette Hospital, Torino, Italy, were included in this study. All patients were affected with histopathologically confirmed primary colon adenocarcinomas representing stage A ($n=6$), B ($n=18$) and C ($n=15$) colon neoplasms according to the Dukes' classification and received no chemotherapy or radiation prior to surgery. Normal colonic mucosa samples were obtained from colonoscopic biopsies performed in 13 patients free of neoplastic or inflammatory diseases. Fresh tumour specimens were obtained after sufficient tissue was secured for histopathology. Informed consent was obtained from all patients. Aliquots were placed in liquid nitrogen prior to mRNA extraction or fixed in formalin and paraffin-embedded for immunohistochemical analysis. Serum samples, collected from patients prior to surgery and from 10 healthy donors, were frozen at −70°C until used.

2.2. Immunohistochemical detection of cytokines

Expression of TGF-β isoforms *in situ* was performed on formalin-fixed, paraffin-embedded sections from primary tumour and nodal metastases using rabbit polyclonal antisera reacting specifically with TGF-β1, TGF-β2, TGF-β3 (epitopes corresponding to carboxy-terminal amino acid sequences of the precursor forms of TGF-β1, TGF-β2, TGF-β3 of human origin, respectively) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and diaminobenzidine tetrahydrochloride as chromogen, as described previously [19]. The specificity of the antisera used was tested by pre-incubation with saturating amounts of the peptides used for immunisation (Santa Cruz Biotechnology), followed by immunostaining of tissue sections. Evaluation of staining patterns was performed using the immunoreactive score (IRS) proposed by Rammele and Stegner [20] in which IRS = SI (staining intensity) × PP (percentage of positive cells). SI was determined as 0, negative; 1, weak; 2, moderate; and 3, strong. PP was defined as 0, negative; 1, 1–20% positive cells; 2, 21–50% positive cells; and 3, 51–100% positive cells. Ten visual fields from different areas of each specimen were chosen at random for IRS evaluation, and the average IRS was calculated.

2.3. Cytokine mapping by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from normal and neoplastic colon tissues was extracted using a commercially available kit based on the single-step Trizol method (Life Technologies, Gaithersburg, MD, USA). Reverse transcription (RT) was performed at 37°C for 1 h, using oligo (dT) primer in a final reaction volume of 20 μl containing 20 U of Superscript II reverse transcriptase, 1×reverse transcriptase buffer, 24 U of RNase inhibitor, and 0.5 mmol/l dNTP mix. For each PCR, 10 μl of first-strand cDNA were added to 20 μl of PCR mix containing 100 ng each of 5′ and 3′ cytokine specific primers and 1 U Taq polymerase. All PCR reagents were purchased from Life Technologies. Human TGF-β1 specific primers were 5′-GCCCTGGACACCAACTATTGC-3′ (sense) and 5′-GCACTTGCAGGAGCGCA-3″ (antisense). PCR conditions were: 4 min at 95°C, 1 min at 58°C, and 35 s at 72°C (32 cycles). The predicted size of TGF-β1 amplimer product was 333 bp. Human TGF-β2 and TGF-β3 specific primers were 5′-AAATGGATACACGAACCCAA-3′ (sense) and 5′-GCTGCATTTGCAAGACTTAC-3′ (antisense); 5′-AAGTGGGTCCATGAACCTAA-3′ (sense) and 5′-GCTACATTTACAAGACTTCAC-3′ (antisense), respectively. PCR conditions were: 4 min at 94°C, 20 s at 51°C, and 25 s at 72°C (32 cycles). The predicted size of both TGF-β2 and TGF-β3 amplimers products was 247 bp. Human β-actin primers and amplification conditions have been described previously [21]. PCR products were analysed by size fractionation, using 2% agarose gels stained with ethidium bromide.

2.4. RNase protection assay (RPA)

Quantitative analysis of TGF-β1, TGF-β2, TGF-β3 mRNA expression was also performed by RNase protection assays using an RNase protection assay (RPA) kit purchased from PharMingen (San Diego, CA, USA).

Briefly, total RNA was isolated as described above. Multiprobe hCK-3, which contains templates for the cytokines TNF-β, LT-β, TNF-α, IFN-γ, IFN-β, TGF-β1, TGF-β2, TGF-β3 and the housekeeping genes L-32 and GAPDH, was labeled with [^{32}P]UTP using T7 RNA polymerase. 3×10^5 cpm of labelled probe was hybridised to 7 μg of total RNA for 16 h at 56°C. mRNA probe hybrids were treated with RNase and phenol-chloroform extracted. Samples were fractioned on a 6% denaturating polyacrylamide sequencing gel. The gel was dried, and the protected fragments were visualised using a Molecular Imager and quantified by Molecular Analyst software analysis (Biorad, Hercules, CA, USA).

2.5. Determination of TGF-β1 and TGF-β2 concentrations in sera

TGF-β1 and TGF-β2 concentrations were determined using ELISA kits available from R&D Systems (Abingdon, UK). The lower threshold of sensitivity of the assays was 50 pg/ml. Both of these kits recognise the activated forms of TGF-β.

2.6. Statistical analysis

In order to compare TGFβ-1, TGFβ-2 and TGFβ-3 values among Dukes' groups, the non-parametric Kruskall–Wallis test was applied; for the multiple comparison in *post hoc* analysis, the Mann–Whitney test with Bonferroni's significance level correction was performed. The correlation between TGF-β's values and Dukes' A, Dukes' B and Dukes' C separately and correlation between mRNA and protein expression were performed with non-parametric correlation Spearmann's R coefficient. The significance level was considered as $P<0.05$.

3. Results

3.1. Immunohistochemical detection of TGF-β isoforms in primary colon carcinoma in situ

Expression of TGF-β proteins in normal and neoplastic colon tissues was determined by immunohistochemistry using isoform-specific antibodies. Results

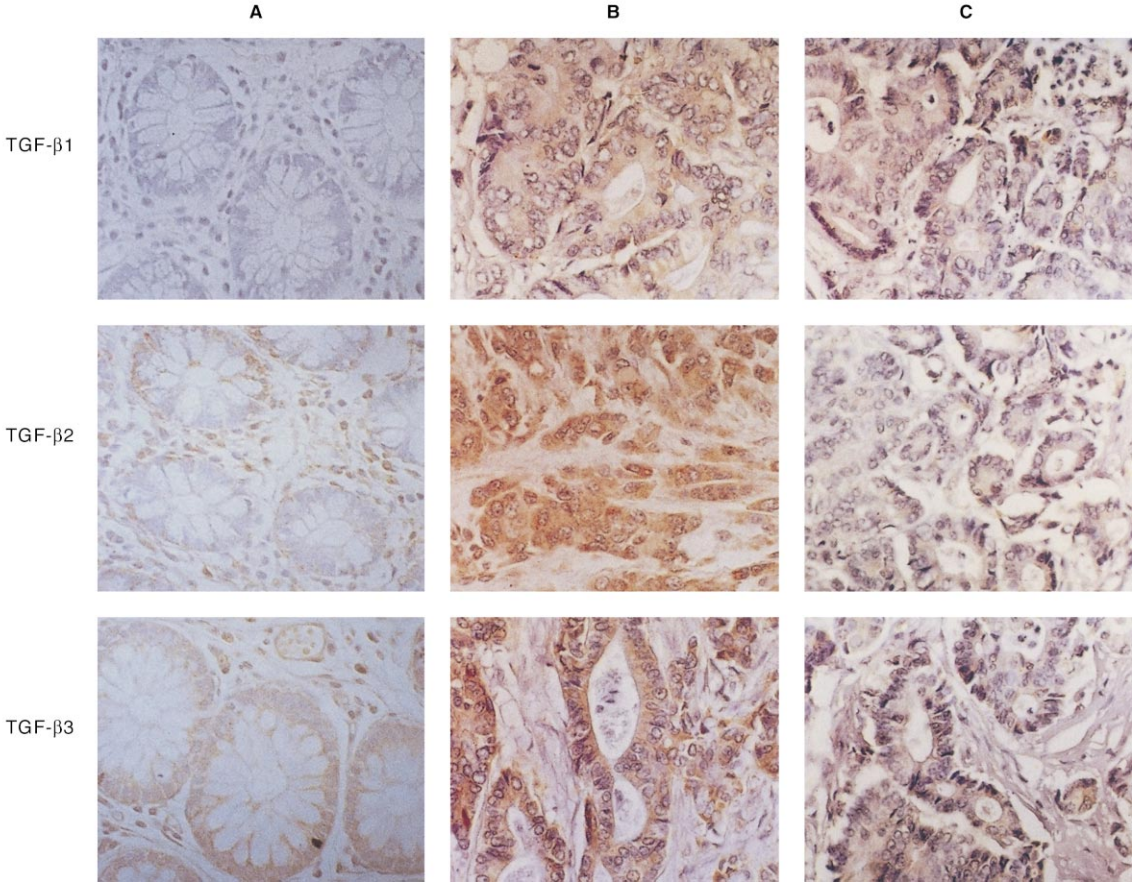

Fig. 1. Expression of TGF-β isoforms in (a) normal and (b) neoplastic colon tissues as determined by immunohistochemistry. Results using a representative example of a Dukes' B colorectal carcinoma are shown. (c) Shows a negative control of neoplastic colon tissue stained with antibodies pre-incubated with saturating amounts of the peptides used for immunisation.

using a representative example of a Dukes' B colorectal carcinoma are shown in Fig. 1. TGF-β1 specific antiserum strongly stained tumour cells as compared with faint staining of normal colon mucosa. TGF-β2 was detected in the cytoplasm of normal columnar but not of crypt cells and was found to be overexpressed by both cell types in neoplastic mucosa. TGF-β3 was expressed in both normal mucosa and tumour tissues, albeit at slightly higher levels in tumour cells. To perform quantitative comparisons of staining patterns in normal and diseased tissues immunoreactive scores were determined according to [20] and subjected to statistical analysis (Fig. 2). This analysis showed significantly elevated levels of TGF-β1 and TGF-β2 but not TGF-β3 in neoplastic mucosa (Fig. 2a). When tumour samples were analysed according to the Dukes' classification, uniform expression of TGF-β3 was observed in tissue specimens representing Dukes' A, B and C. By contrast, staining intensity with TGF-β1 and TGF-β2 antibodies gradually increased in successive stages of colorectal tumour progression. This trend was statistically significant in the case of TGF-β1 but not TGF-β2 (Fig. 2b). In addition, when intensity of staining for individual TGF-β isoform was correlated within Dukes' stages, relationships were observed with significant R values only between TGF-β2 and TGF-β3 on Dukes B ($R=0.504$, $P=0.03$) and Dukes C ($R=0.703$, $P=0.002$) (Fig. 3). No correlation was found between intensity of staining and differentiation state of the tumour (data not shown). In summary, colorectal carcinomas *in situ* overexpress two of the three known TGF-β isoforms in a tumour-associated manner.

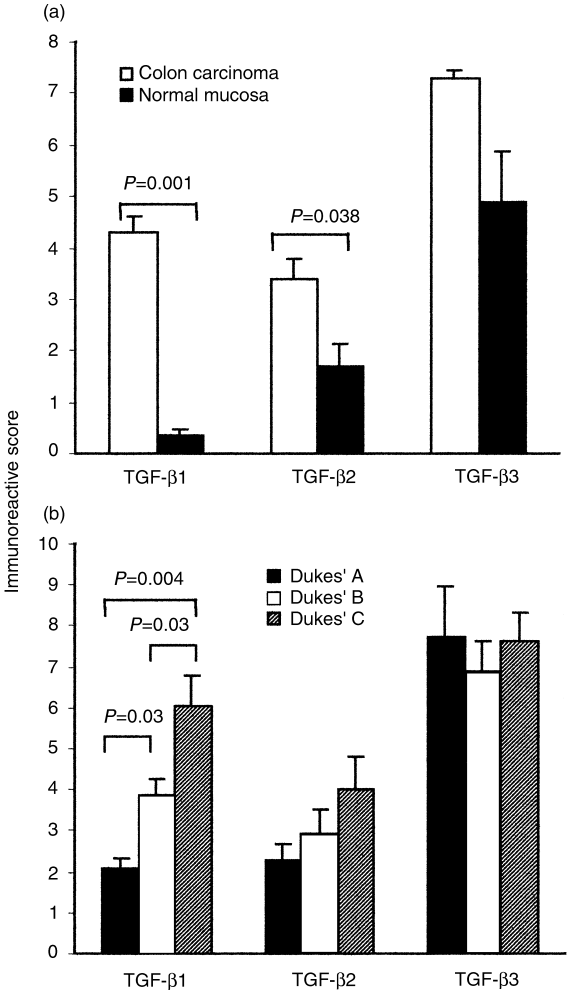

Fig. 2. Quantitative analysis of immunohistochemical staining of normal ($n=10$) and neoplastic colonic mucosa ($n=39$) with TGF-β isoform specific antibodies. The immunoreactive scores were obtained as described in Patients and Methods: (a) statistically significant differences between immunoreactive scores of tumour and normal tissues were found for TGF-β1 ($P=0.001$) and TGF-β2 ($P=0.038$); (b) subsequent stages of colorectal tumour progression (Dukes' A $n=6$; Dukes' B, $n=18$; Dukes' C, $n=15$) were characterised by statistically significant increases in TGF-β1 immunoreactive scores.

3.2. *Immunohistochemical detection of TGF-β isoforms in lymph nodes with metastatic disease*

To assess changes in TGF-β expression during metastatic spread of colon carcinoma cells, we determined, by immunohistochemistry, expression profiles of all three isoforms in primary sites and lymph node metastases from the same patients; a representative case (T3 N2 M0 G2) is shown in Fig. 4. Generally, TGF-β1, TGF-β2 and TGF-β3 immunoreactivities were comparable between tumour cells in lymph nodes and in primary tumours.

3.3. *Expression of TGF-β mRNAs in colorectal carcinoma* in situ

To confirm the results obtained by immunohistochemical detection of TGF-β proteins, we determined expression of TGF-β mRNAs by RT-PCR and RPA. As shown in Fig. 5, amplimers corresponding to all three TGF-β isoforms were generated from most ($n=30$) tumour samples and all normal mucosa specimens ($n=10$). Notably, normal mucosa specimens demonstrated heterogeneous expression of TGF-βs, particularly TGF-β2, which was weak or absent in five of 10 samples analysed. In comparison, mRNA expression of all three TGF-β isoforms appeared to be homogeneous in all tumour specimens. To provide a better quantitative measure of TGF-β mRNA expression, we performed RPAs (Fig. 6). This analysis showed significantly higher expression of TGF-β1 and TGF-β2 mRNAs in colon carcinoma as compared to control tissues (Fig. 6b, $P=0.043$ and $P=0.028$, respectively). A similar trend was observed for TGF-β3, although it did not reach statistical significance due to significant scattering of data in the malignant samples. When the analysis was restricted to the malignant cases, a highly

significant difference was observed only between TGF-β1 and TGF-β2 mRNA levels ($P=0.007$) In addition, there was a significant positive correlation between TGF-β isoform mRNA expression (TGF-β1 versus TGF-β2: Spearman correlation coefficient = 0.855, $P=0.02$; TGF-β1 versus TGF-β3: Spearman correlation coefficient = 0.939, $P=0.0001$; TGF-β2 versus TGF-β3: Spearman correlation coefficient = 0.794, $P=0.006$).

3.4. TGF-β levels in sera of patients with colon carcinomas

Previous work by us and others has shown that over-expression of various cytokines including interleukin-10 and TGF-β by tumour cells is associated with increased levels of these cytokines in patients' sera [11,14,15]. We, therefore, determined by isoform-specific ELISA the levels of the TGF-β1 and TGF-β2 active forms in colon carcinoma patients ($n=10$) relative to normal controls ($n=7$). A similar analysis of TGF-β3 serum levels was precluded by the lack of a sensitive TGF-β3-specific ELISA. Consistent with the idea that tumour-derived TGF-β is released into the circulation of tumour patients, we observed significantly elevated levels of both, TGF-β1 and TGF-β2 in cancer patients (Fig. 7). Statistically significant correlations were observed between expression of TGF-β1, TGF-β2 and TGF-β3 at mRNA and protein levels within tumours and TGF-β1, and TGF-β2 serum levels (Table 1).

3.5. Correlation between TGF-β isoform expression and clinical outcome

In 29 patients, disease progression was followed for 3 years after primary diagnosis (Table 2). When disease progression was compared to TGF-β isoform expression in primary tumours, a linear relationship ($R=0.982$) was revealed between expression of TGF-β1

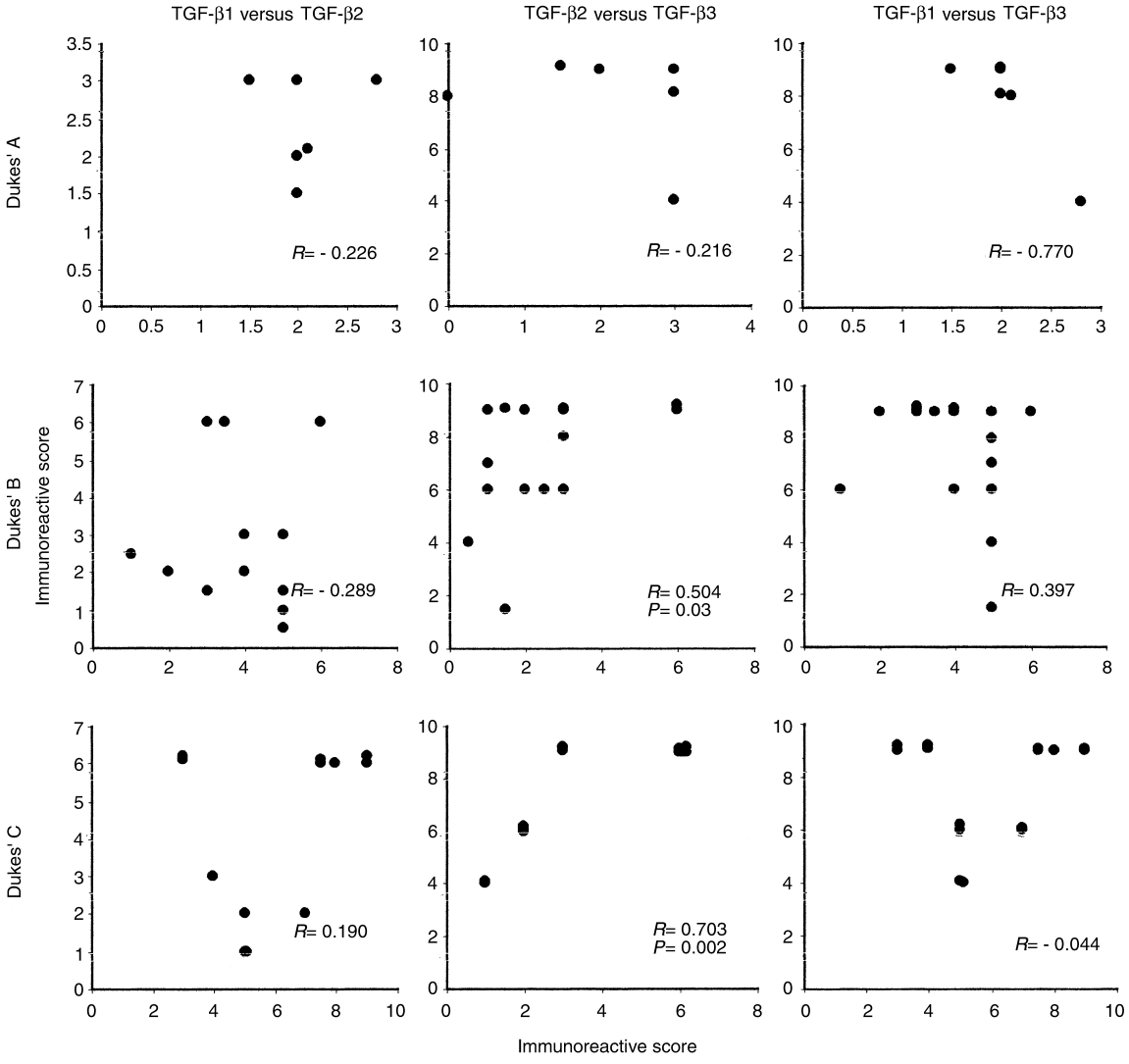

Fig. 3. Correlation between the intensity of reactivity of individual TGF-β isoforms within the Dukes' stages.

and progression but not for the other two TGF-β (Fig. 8).

4. Discussion

The present study demonstrates that colon carcinoma progression is associated with increased expression of TGF-β1 and TGF-β2 in tumour cells relative to normal mucosa. TGF-β3 protein was expressed in normal mucosa and slightly upregulated in neoplastic cells. The elevated expression of TGF-β1 and TGF-β2 mRNAs and proteins in tumour cells was associated with elevated levels of both isoforms in patients' sera.

These results corroborate and extend earlier observations on TGF-β expression in colon cancer. Several groups have shown TGF-β mRNA or protein overexpression in neoplastic as compared with normal colonic tissue [15–17,22,23]. These earlier studies focused primarily on expression of only one TGF-β isoform (TGF-β1) in colon carcinoma and generally reported elevated levels of TGF-β1 in neoplastic colon mucosa [24,25]. For example, Friedman and colleagues [16] and Tsushima and colleagues [26] described that, in colorectal carcinomas, TGF-β1 expression correlated significantly with disease progression to metastasis. Our results confirm these earlier observations. Similarly, we observed that successive stages of colon carcinoma pro-

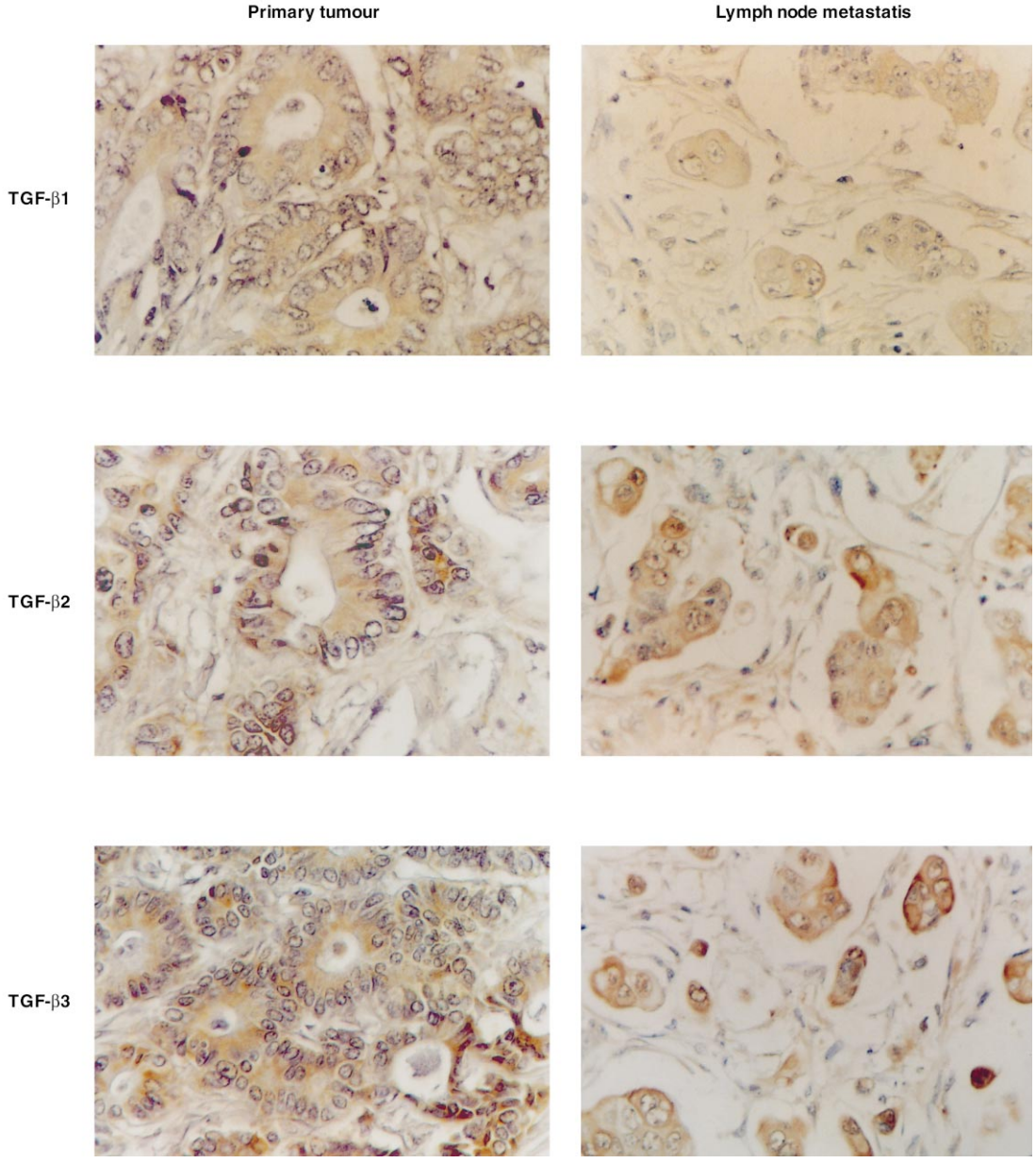

Fig. 4. Expression of TGF-β isoforms in primary tumour and nodal metastasis as determined by immunohistochemistry. Results using a representative example of a Dukes' C colorectal carcinoma are shown.

gression are characterised by a gradual increase in TGF-β1 immunoreactivity with the most intense staining observed in Dukes' C primary tumours. Our finding that plasma levels of TGF-β1 are elevated in tumour patients is consistent with very recently published results of Shim and co-workers [25] of elevated plasma levels of TGF-β1 in colon carcinoma patients. An earlier study using a syngeneic colon carcinoma in mice reported a positive correlation with tumour size and TGF-β concentration in plasma [24]. We observed intense immunostaining of neoplastic mucosa cells with TGF-β1 antibodies consistent with the view that at least part of this activity derives from the tumour cells. However, it can not be excluded that, in addition to tumour cells, other cell types contribute to the high serum TGF-β1 levels in patients. Irrespective of its source, TGF-β1 in peripheral blood is likely to exert systemic effects, for example on the immune system.

To our knowledge, production of TGF-β2 and TGF-β3 by colon carcinomas *in situ* has not been studied in detail previously. We describe overexpression of TGF-β2 in tumour specimens when compared with normal mucosa. Like TGF-β1, TGF-β2 is most strongly expressed at later stages of tumour progression (Dukes' B, C; Fig. 2) when compared with Dukes' A tumours, although this trend was not statistically significant. In contrast to TGF-β1, immunoreactive TGF-β2 was also detected in some normal specimens. In addition, expression of TGF-β2 was highly heterogeneous when assessed by RT-PCR in normal mucosa specimens. This conclusion is supported by the absence of amplification products detectable in ethidium bromide-stained gels in four of the 10 normal samples tested. As in the case of TGF-β1, plasma levels of TGF-β2 were elevated in tumour patients as compared with normal individuals.

The pattern of TGF-β3 expression in colonic mucosa is distinct from that observed for the other two TGF-β isoforms. This conclusion is supported by our finding that immunoreactivity with TGF-β3-specific antibodies was high in normal mucosa and not statistically different between normal mucosa and tumour sections (see Fig. 2a). In addition, tumour-associated expression of immunoreactive TGF-β3 was uniform across successive stages of tumour progression. Similarly, TGF-β3 mRNA levels were not significantly different between normal and tumour samples although tumour tissues generally expressed slightly higher levels as determined by RPA.

In recent years, knock-out mice for the three TGF-β isoforms have been generated. Targeted disruption of the mouse TGF-β1 gene results in multifocal inflammatory disease [27], indicating a prominent role of TGF-β1 in suppressing excessive inflammation. TGF-β3 −/− mice have a comparatively mild phenotype characterised by localised defect in epithelial cell differentiation which manifests itself in failure of the palatal shelves to fuse leading to cleft palate [28]. The most severe phenotype is caused by ablation of TGF-β2 expression which results in multiple developmental defects affecting the skeleton, heart, eyes, ears and urogenital tract [18]. Taken together, these studies suggest tissue-specific, non-redundant roles of different TGF-β isoforms in normal development. It is possible that they similarly play divergent roles in tumour development that cannot be compensated by other isoforms. Recent studies have confirmed that TGF-β overexpression is not only associated with progression of colorectal carcinomas [29] but that TGF-β signalling is required for dissemination and metastasis of colorectal carcinoma cells in experimental animals [30]. Our findings in support of different expression patterns of TGF-β isoforms in colon carcinoma progression provide a baseline to the investigation of possibly distinct roles of TGF-β1, TGF-β2 and TGF-β3 in these processes.

In addition, genetic studies have clearly revealed a role for TGF-β1 and its receptors in embryonic

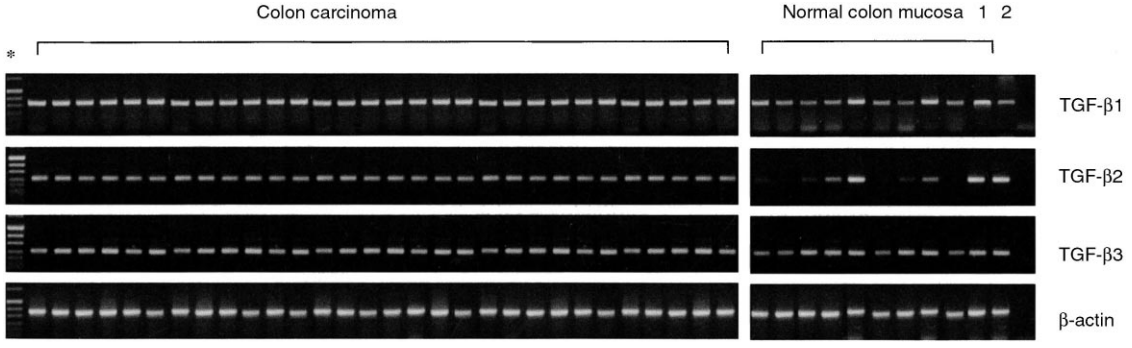

Fig. 5. Expression of TGF-β mRNA in neoplastic and normal colon mucosa as determined by RT-PCR analysis. Amplimers for TGF-β1, TGF-β2 and TGF-β3 of the predicted size (333 bp, 247 bp, 247 bp, respectively) were generated using specific primers using RNA from colon carcinoma patients ($n = 30$) and subjects free of neoplastic or inflammatory diseases ($n = 10$). Lane 1 shows the amplification product for all three TGF-β isoforms using DLD-1 colon carcinoma cell line as a positive control. Lane 2 shows the absence of amplification products in the absence of a cDNA template. Molecular weight markers are marked by *.

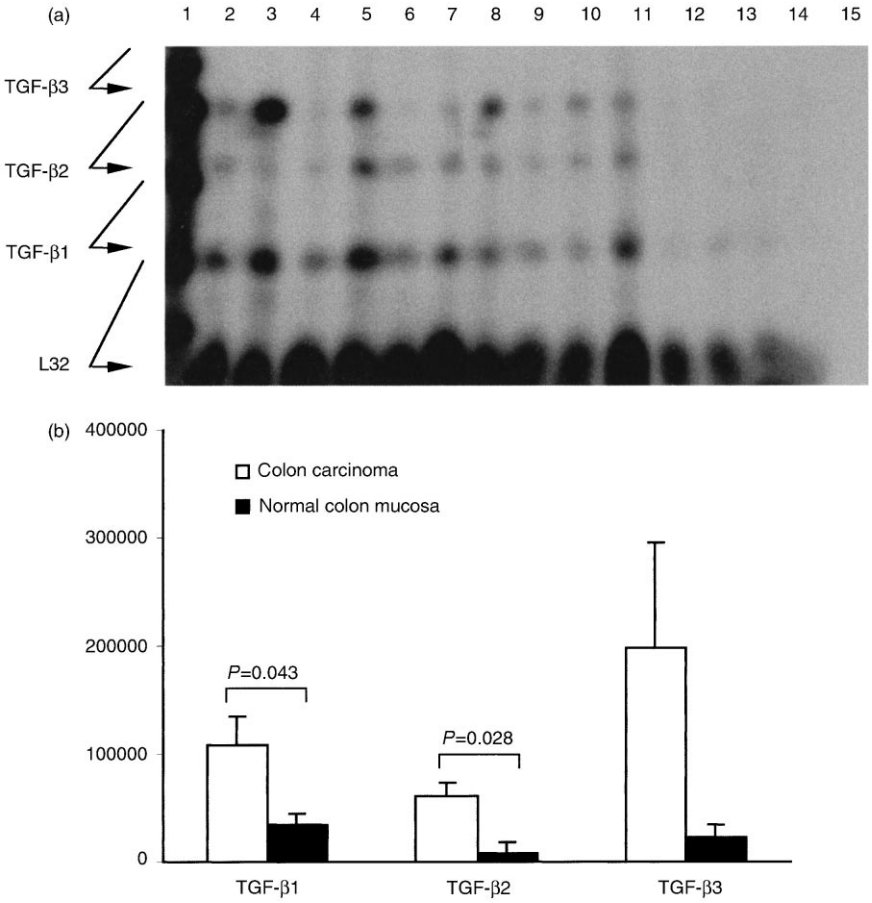

Fig. 6. Representative quantitative analysis of steady-state TGF-β isoform mRNA expression in neoplastic ($n=10$) and normal ($n=3$) colon mucosa specimens. Total RNA was isolated and analysed by RNase protection assay as described in Patients and Methods. The arrows in (a) denote the protected RNA species corresponding to the isoforms indicated. Free RNA probe is shown in lane 1. Lanes 2, 5, 8, 10, 11: Dukes' C colon carcinomas. Lanes 3, 6, 7, 9: Dukes' B colon carcinomas. Lane 4: Dukes' A colon carcinoma. Lanes 12–14: normal colon mucosa. Lane 15: yeast tRNA, used as negative control. (b) shows comparative densitometric analysis of hybridisation signals obtained using normal and malignant samples ($n=30$ colon carcinomas and $n=10$ normal colon mucosae). Statistically significant differences between datasets are indicated by P values.

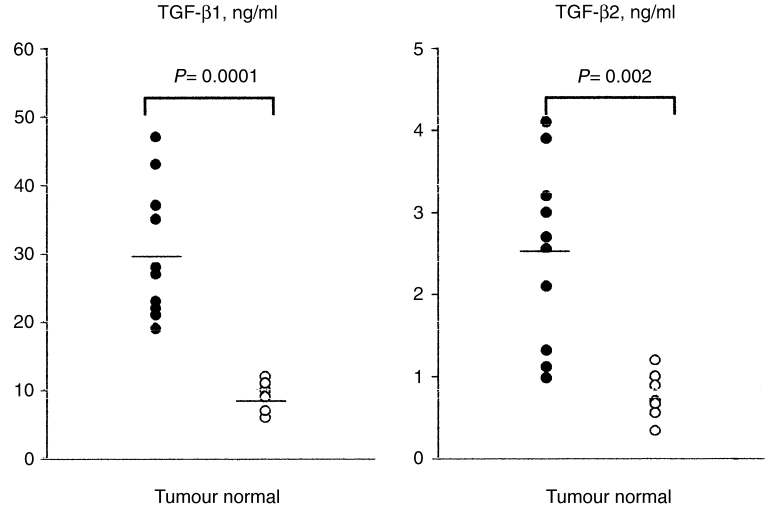

Fig. 7. Detection of TGF-β1 and TGF-β2 in sera obtained from normal donors ($n=7$) and colon carcinoma patients ($n=10$) as determined by ELISA. Bars within datasets represent mean values. Statistically significant differences between datasets are indicated by P values.

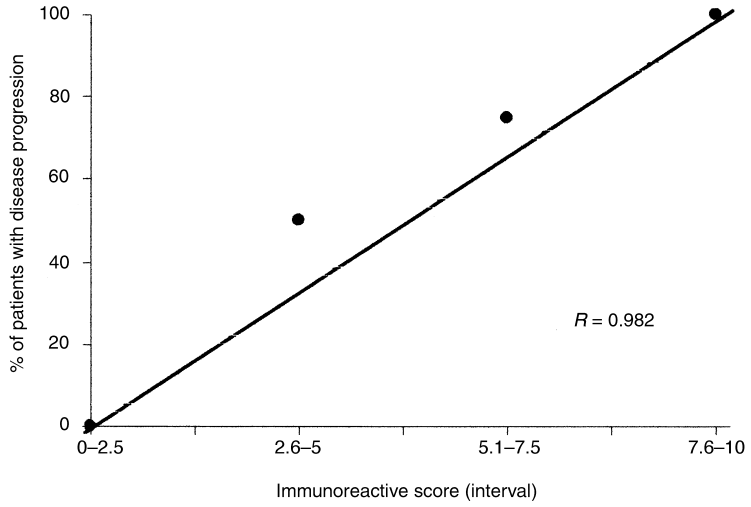

Fig. 8. Correlation between TGF-β1 immunoreactivity in primary tumour and disease progression in 29 patients whose disease recurred within 3 years or who remained cancer-free for 3 years.

Table 1
Correlation between TGF-β isoform mRNA expression and protein abundance *in situ* and in serum

	Immunoreactive score			Serum levels	
	TGF-β1	TGF-β2	TGF-β3	TGF-β1	TGF-β2
mRNA expression					
TGF-β1	$R=0.902$ ($P=0.0001$)	$R=0.203$ (NS)	$R=-0.902$ (NS)	$R=1.000$ ($P=0.0001$)	$R=0.830$ ($P=0.003$)
TGF-β2	$R=0.671$ ($P=0.034$)	$R=0.260$ (NS)	$R=0.0001$ (NS)	$R=0.855$ ($P=0.002$)	$R=0.988$ ($P=0.0001$)
TGF-β3	$R=0.823$ ($P=0.003$)	$R=0.285$ (NS)	$R=0.055$ (NS)	$R=0.939$ ($P=0.0001$)	$R=0.782$ ($P=0.008$)

R, non-parametric correlation Spearmann's coefficient; NS, not significant

Table 2
Intensity of staining in primary tumour in a subset of patients ($n=29$) who disease recurred within 3 years or who remained disease free for 3 years

Immunoreactive score	No. of patients	
	Recurrence	No recurrence
TGF-β1		
0–2.5	0	4
2.6–5	2	2
5.1–7.5	6	2
7.6–10	13	0
TGF-β2		
0–2.5	2	2
2.6–5	2	2
5.1–7.5	4	3
7.6–10	8	6
TGF-β2		
0–2.5	2	2
2.6–5	3	2
5.1–7.5	5	2
7.6–10	6	7

vascular assembly and in the establishment and maintenance of vessel wall integrity. Stable transfection with TGF-β1 has been shown to confer a growth advantage on Chinese hamster ovary cells in nude mice, and this was associated with the development of a metastatic phenotype [31]. Tumour growth in this system was accompanied by an increase in capillary density, and local administration of neutralising antibodies to TGF-β1 reduced both neoangiogenesis and tumour development [32]. Therefore we can postulate that the overproduction of TGF-β1 by colon carcinoma cells may participate in tumour development *in vivo* by promoting both the neoangiogenesis at the primary tumour site and the following metastatic progression.

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