CANCER

Associated changes of lipid peroxidation and transforming growth factor β 1 levels in human colon cancer during tumour progression

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Gut 2002;50:361–367

Background: During neoplastic progression, alterations in transforming growth factor β 1 (TGF- β 1) dependent control of cell growth may be an important mechanism of selective proliferation of transformed cellular clones. Defective regulation of TGF- β 1 receptors has been reported to occur in a number of human malignant tumours while little is known of the actual levels of this growth inhibitory cytokine in cancer. On the basis of the demonstrated ability of major lipid peroxidation products such as 4-hydroxynonenal to modulate TGF- β 1 expression and synthesis, we speculated that decreased lipid oxidation, as frequently observed in neoplastic tissues, would contribute to the selective promotion of tumour growth through decreased expression of the cytokine within the tumour mass.

Aims: To seek a possible association between steady state levels of major aldehydic end products of lipid peroxidation and TGF- β 1 content in human colon cancer at different stages of growth.

Patients and methods: Tissue biopsies from 15 adult patients with colon adenocarcinoma of different TNM and G stagings were compared with regard to lipid peroxidation aldehydes and net TGF- β 1 levels. For a more comprehensive analysis, cytokine type I and II receptors were measured in tumour biopsies. In one set of experiments, to support the conclusions, the apoptotic effect of TGF- β 1 was evaluated in a human colon cancer cell line, CaCo-2, retaining receptor changes consistent with those observed in cancer patients.

Results: With the exception of two extremely advanced cases (T4/G3) in which tissue levels of lipid peroxidation were within the normal range, 4-hydroxynonenal was significantly decreased in all other cancer specimens. Consistent with lipid peroxidation levels, TGF- β 1 protein was markedly decreased or even negligible compared with the corresponding normal tissue surrounding the tumour in all tested biopsies except for the two T4/G3 colon cancers in which cytokine content was again within the normal range. As regards TGF- β 1 receptors, both in tumour sections and CaCo-2 cells, downregulation was greater for TGF- β 1 receptor I than for receptor II. Of note, in CaCo-2 cells, incubation with appropriate doses of TGF- β 1 led to marked nuclear fragmentation and apoptosis.

Conclusions: Evasion of human colon cancer cells from TGF- β 1 mediated growth inhibition appears to be due not only to downregulation of TGF- β 1 receptors, which is inconsistent and unrelated to cancer development, but also to the constant low concentration of this cytokine in the tumour mass. The associated levels of lipid peroxidation aldehydes, much lower than in control tissue, probably represent a lower stimulus for TGF- β 1 production in the neoplastic area and thus a favourable condition for neoplastic progression.

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Accepted for publication 5 June 2001

Transforming growth factor β 1 (TGF- β 1) is a multipotent cytokine recognised as playing an important role in regulating cell growth and development.^{1,2} Two other isoforms (β 2, β 3) with significant sequence homology and similar functions have also been described in mammalian tissues but are much rarer.^{3,4}

TGF- β 1 is secreted by most mammalian cells in a latent non-active complex from which a 25 kDa bioactive dimer can be released. The latter binds through the ubiquitous type I (TGF- β 1-RI) and type II (TGF- β 1-RII) receptors⁵ to a wide variety of cell types, inducing a number of effects such as immunosuppression, extracellular matrix deposition, cell cycle arrest and cell differentiation, apoptosis of normal and neoplastic cells.⁴

TGF- β appears to be a potent growth inhibitor for most cells, including epithelial, endothelial, and lymphatic cells (see Grande⁴ for a comprehensive review). Consequently, disruption of the TGF- β growth inhibitory autocrine/paracrine loop should crucially favour uncontrolled cell proliferation and transformation. This hypothesis is at present mainly supported by the frequent finding of defective alterations in the

TGF- β receptor system in cancers of the stomach and colon,⁶ prostate,⁷ breast,⁸ and lung.⁹ Furthermore, expression of receptor type II has been found to be low or even absent in colon and lung cancer cells with microsatellite instability, indicating a close association between impairment of the TGF- β receptor system and genetic abnormalities.^{10–13}

However, not all individuals bearing cancers of the above mentioned types show impairment of the TGF- β receptor system in the tumour mass. For example, in patients with adenocarcinoma of the lung, Kim *et al* reported decreased TGF- β 1-RII levels in only 25% of the group⁹ while in patients with colorectal cancer Matsushita *et al* showed downregulated

Abbreviations: TGF- β 1, transforming growth factor β 1; TGF- β 1-RI, TGF- β 1 receptor type I; TGF- β 1-RII, TGF- β 1 receptor type II; MDA, malonaldehyde; HNE, 4-hydroxynonenal; DMEM, Dulbecco's modified Eagle's medium; TBA, thiobarbituric acid; PBS, phosphate buffered saline; DAPI, 4,6-diamidino-2-phenylindole; TUNEL, TdT mediated dUTP nick end labelling.

Figure 1 Characterisation of apoptosis in the CaCo-2 cell line using the TdT mediated dUTP nick end labelling (TUNEL) test. (A) Untreated CaCo-2 cells; (B) CaCo-2 cells treated with 10 ng/ml transforming growth factor β 1 (TGF- β 1) for 48 hours. Fluorescence was detected by a laser scanning confocal microscope (Zeiss): the laser band of excitation was 488 nm with a 505 nm long pass filter emission; the lens used was a plan neofluar 40 $\times$ /0.75. The image dimension was 325 μ m $\times$ 325 μ m $\times$ 10 μ m, and the unit scale used was 0.64 μ m². The image was elaborated using a LSCM 510 Image Examiner Program (Zeiss). Exciting light intensity, black level, and photomultiplier gain were adjusted on control specimens; the same setting was employed for scanning of experimental material.

expression of TGF- β 1-RI and/or RII receptors in 38% of the group, the receptor system being normally expressed in the remainder who had similar TNM stages.¹⁴

Hence TGF- β receptor deficiency is certainly an important mechanism of tumour progression but it does not appear to be absolutely necessary for this process to develop. Some questions may be posed: are cells with low RI and/or RII mRNA levels completely insensitive to the antiproliferative effect of TGF- β ? Is there any change in the net amount of this cytokine within the tumour mass independent of changes in the TGF- β receptor system? With regard to the first question, *in vitro* data on preservation of a significant apoptotic effect of TGF- β 1 on human colon cancer cells (CaCo-2 cell line), despite low levels of TGF- β 1-RI and RII, are reported here. In relation to the second question, we previously demonstrated that a major end product of membrane lipid peroxidation, 4-hydroxynonenal (HNE), may contribute significantly towards upregulating both TGF- β 1 expression and its synthesis.¹⁵ On the other hand, low lipid peroxidation has consistently been demonstrated in biomembranes from a variety of experimental cancer cell lines and carcinogenesis models (see Dianzani¹⁶ for a comprehensive review). Thus if a true association exists between the extent of lipid peroxidation reactions and TGF- β 1 expression, one should also expect a decrease in the level of this cytokine in those types of human cancer found to be resistant to lipid oxidation, with further advantage for cancer cell dedifferentiation and growth. Indeed, in 13 of 15 patients with colon cancer, a significant decrease in the lipid peroxidation product HNE was found in the tumour mass, and it was consistently associated with a net decrease in TGF- β 1 protein. Only in two extremely advanced cases (G3/T4) were both lipid peroxidation indices and cytokine levels within the control range.

METHODS

Cell culture

CaCo-2 human colorectal carcinoma cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) from Gibco BRL Life Technologies Italia Srl (S Giuliano Milanese, Milano, Italy) containing 862 mg/l L-alanyl-L-glutamine (Glutamax-I), 4500 mg/l glucose, and 110 mg/l sodium pyruvate, supplemented with 20% fetal bovine serum (Gibco BRL, Life Technologies Italia Srl), and antibiotics/antimycotics (100 units/ml penicillin, 0.1 mg/ml streptomycin, and 250 ng/ml amphotericin B from

Sigma Aldrich Italia Srl, Milano, Italy). Cells were cultured in 25 cm² flasks at 37°C in a humidified atmosphere of 5% CO₂ in air and five days after plating (about 70% confluency) were used for the different experiments. At the end of the treatments, CaCo-2 cells were harvested by trypsinisation with trypsin-EDTA (2.5 g/l—1 g/l in 0.9% sodium chloride) (Sigma Aldrich Italia Srl) and resuspended at 200 000 cells/ml in serum and phenol red free DMEM. A total of 80 000 cells were collected by cytocentrifugation at 30 *g* for seven minutes (Cytospin I; Shandon Inc., Pittsburgh, USA).

Tumour bearing patients

Fifteen adult patients (eight women, seven men) affected by colon adenocarcinoma were recruited from San Luigi Gonzaga Hospital (Orbassano, Torino, Italy). Mean age of the patients was 60 (10) years.

The different stages of tumour malignancy were defined both in terms of the TMN classification system (T2, five cases; T3, eight cases; T4, two cases) and the histological grading of atypia (G2, 11 cases; G3, four cases).

Tissue sample processing

The surgically removed segment of colon of each patient was divided into two sections: one zone directly involved in the tumour mass and another distal from the tumour site (normal tissue). Part of the tumour biopsy was embedded in paraffin and part was directly frozen in liquid nitrogen and stored at -80°C until use.

Biochemical markers of lipid peroxidation

The presence of oxidative damage was assessed in 10% (w/v) tissue homogenates prepared from biopsies in 0.1 M Tris HCl buffer (pH 7.4) using a Polytron apparatus (Kinematika GmbH, Luzerna, Switzerland): protein-aldehyde adducts were determined in homogenates as fluorescence exhibited by interaction between protein amino functions and malonaldehyde (MDA) or HNE at wavelengths of 390/460 nm and 355/460 nm, excitation/emission respectively.^{17,18}

MDA levels were also evaluated in 10% (w/v) tissue homogenate specimens using the thiobarbituric acid (TBA) reaction¹⁹: homogenates were incubated in a thermostatic shaking bath at 37°C for one hour before evaluation.

Western blot analysis of TGF- β 1 tissue levels

Tissues were homogenised and after evaluation of protein content by Lowry assay,²⁰ samples were normalised—that is, adjusted to achieve an equal amount of protein. For immunoblot analyses, 40 μ g of total protein were denatured in Laemmli buffer,²¹ loaded on the same 12% polyacrylamide sodium dodecyl sulphate gel and subjected to electrophoresis under reducing conditions. Coomassie blue staining was used to verify appropriate protein normalisation in the electrophoretic run. Proteins were transferred onto nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech GmbH, Germany). Unspecific binding was blocked with 5% (w/v) non-fat dried milk in Tris sodium buffer (TBS)-Tween (50 mM Tris HCl, pH 7.4, containing 200 mM NaCl, 0.05% v/v Tween 20). Blotted membranes were incubated with a polyclonal TGF- β 1 primary antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) followed by incubation with peroxidase conjugated polyclonal antirabbit secondary antibodies (Santa Cruz Biotechnology). The immunoblots were developed with the ECL detection system (Amersham Pharmacia Biotech GmbH, Germany) following the manufacturer's directions.

Immunocytohistochemical receptor analysis

TGF- β 1 receptors were visualised both on the CaCo-2 cell line and on tissue sections from patients affected by colon adenocarcinoma.

Figure 2 Immunohistochemical analysis of types I and II transforming growth factor β 1 (TGF- β 1) receptors in colon cancer versus normal mucosa in a representative patient (reported as CP in table 1). Receptors were visualised using an appropriate purified rabbit polyclonal antibody; colour was developed as described in materials and methods. Normal colon mucosa: expression of type I (A) and type II (B) TGF- β 1 receptors (100 $\times$ magnification). Colon cancer: expression of type I (C, 40 $\times$; E, 250 $\times$) and type II (D, 40 $\times$; F, 250 $\times$) TGF- β 1 receptors.

CaCo-2 cells were plated and harvested (see cell culture above). After cytocentrifugation the slides were fixed in para-formaldehyde solution (4% in phosphate buffered saline (PBS), pH 7.4) for 10 minutes and permeabilised with 0.1% Triton X-100 in 0.1% sodium citrate for five minutes at 4°C. Paraffin was removed from the embedded tissue sections through consecutive passages with pure xylol, 99% and 95% ethanol, and water. For antigen retrieval the samples were sunk in 10 mM sodium citrate buffer (pH 6.0) and heated in a microwave oven (for three minutes at 750 W and twice for two minutes at 350 W).

In both CaCo-2 cells and tissue specimens, endogenous peroxidase was inhibited by incubation in 0.6% H_2O_2 for 25 minutes. After preincubation with bovine serum albumin in TBS (0.1 % w/v) containing 30% normal porcine serum (v/v) for 20 minutes at room temperature to block non-specific binding, slides were incubated in the presence of the following polyclonal rabbit antibody: 4 μ g/ml anti-TGF- β 1-RI (L-21) or 8 μ g/ml anti-TGF- β 1-RII (T-19) (Santa Cruz Biotechnology). Incubation was performed in a humidified chamber at 4°C overnight. Negative controls were incubated overnight in the presence of 30% normal porcine serum (v/v) alone.

In order to block endogenous avidin and biotin, slides were preincubated for 10 minutes with 0.005% avidin and for 10 minutes with 0.005% biotin (Dako Biotin Blocking System; Dako SpA, Milano, Italy). After this treatment samples were incubated with polyclonal pig antirabbit biotinylated immunoglobulins (1:200 dilution v/v) (Dako SpA), and then with

biotin-avidin-peroxidase reagent (ABC complex-HRP; Dako SpA) at room temperature for 30 minutes. The bound immunocomplex was visualised by incubation with 0.6% 3,3'-diaminobenzidine tetrahydrochloride in TBS containing 0.003% H_2O_2 for 5–10 minutes. All incubations were preceded by three washes with TBS (Tris NaCl buffer, 50–150 mM, pH 7.6) for five minutes.

Sections were lightly counterstained with Mayer haematoxylin.

Morphological detection of apoptosis induced by TGF- β 1

To induce apoptosis, five days after plating (about 70% confluency) CaCo-2 cells were treated with two repeated doses of TGF- β 1 (5 ng/ml) (Boehringer Mannheim, Roche Diagnostics, Monza, Milano, Italy) at time zero and after 24 hours.

Twenty four hours after the second TGF- β 1 treatment, cells were harvested from tissue flasks and cytocentrifuged at 30 g for seven minutes (to obtain 80 000 cells/slide).

To detect nuclear staining by 4,6-diamidino-2-phenylindole (DAPI), after collection by cytocentrifugation slides were fixed in ethanol:ether 1:1 (v:v) for 10 minutes, incubated at 37°C for 10 minutes in DAPI solution (1 μ g/ml of absolute methanol, final concentration), and washed with PBS and 96% ethanol. Analysis of nuclear morphology changes was conducted using a fluorescent microscope with an ultraviolet filter, 630 $\times$ magnification.

Table 1 Clinical profiles of patients affected by colon adenocarcinoma and corresponding immunohistochemical expression of transforming growth factor β 1 receptor type I (TGF- β 1-RI) and type II (TGF- β 1-RII)

Patient	Sex	Age (y)	pTNM	Histology (grading)	TGF- β 1-RI*		TGF- β 1-RII*	
					N	Ca	N	Ca
BB	F	46	T3N2M1	G2	54	100	100	100
CV	M	50	T3N0M0	G2	97	78	93	100
DP	F	46	T2N0M0	G2	88	59	99	100
MA	M	68	T3N0M0	G2	100	98	100	100
PR	F	61	T3N2Mx	G2	51	66	82	100
TE	M	80	T2N0M0	G2	10	30	8	9
BM	M	59	T3N0M0	G2	72	100	100	100
CA	M	56	T2N0M0	G2	100	100	100	90
DG	F	56	T2N0M0	G2	100	14	100	100
PI	F	72	T2NxM0	G2	100	58	88	50
CP	M	69	T3N1Mx	G2	94	6	100	97
CG	M	59	T3N0M0	G3	90	100	100	100
MB	M	90	T3N0M0	G3	100	6	100	100
GS	F	54	T4N2M1	G3	100	100	100	67
TZ	F	72	T4N1M0	G3	100	2	100	50

N, normal colorectal tissue; Ca, cancerous tissue.

pTNM classification: pT, extent of the primary tumour (T2, tumour invading muscularis propria; T3, tumour invading subserosa; T4, tumour directly invading other organs and/or visceral peritoneum); pN, regional lymph node metastasis (Nx, local lymph nodes not evaluable; N0, local lymph nodes without metastasis; N1, metastasis in 1–3 local lymph nodes; N2, metastasis in four or more local lymph nodes); pM, distant metastases (Mx, metastases not evaluable; M0, no metastasis; M1, presence of metastases).

Histological tumour deviation was graded as follows: G2, moderate; G3, intense.

*The number of positive TGF- β 1-RI and TGF- β 1-RII cells are expressed as a percentage of the total cells examined at 250 $\times$ magnification (for each slide, 20 non-consecutive randomised histological fields were evaluated).

Nuclear fragmentation was also detected by the TdT mediated dUTP nick end labelling (TUNEL) technique. Cells were fixed in paraformaldehyde solution (4% in PBS, pH 7.4) for 10 minutes and permeabilised with 0.1% Triton X-100 in 0.1% sodium citrate for two minutes at 4°C. The staining reaction was performed using the “in situ cell death detection kit” from Boehringer Mannheim (Roche Diagnostics SpA, Monza, Italy) and visualised by fluorescein at 488 nm excitation with 505 nm long pass filter emission using a laser scanning confocal microscope (LSCM Zeiss, Germany). The objective used was 40 $\times$, the dimensions of the optical sections were 325 μ m $\times$ 325 μ m $\times$ 10 μ m, and the unit scale was 0.64 μ m².

All slides were sealed with Vectashield H-1000 mounting medium (Vector Lab. Inc., Burlingame, California, USA).

Statistical analyses

Data are mean (SD). The statistical significance of differences between experimental groups was assessed using the Student's *t* test.

RESULTS

TGF- β 1 induced apoptosis in human colon cancer CaCo-2 cell line

The immunocytochemical semiquantitative analysis for the TGF- β receptor system in the CaCo-2 cell line performed in our laboratory showed low levels of both TGF- β 1-RI and TGF- β 1-RII receptors: only 30% of the randomly examined cancer cells were immunostained when antibodies against type I receptor were employed while about 60% were immunostained for TGF- β 1-RII receptors (data not shown). Of note, such pronounced TGF- β 1 receptor deficiency was not sufficient to abolish the proapoptotic effect of the cytokine. In fact, treatment of CaCo-2 cells with an appropriate concentration of TGF- β 1 (two doses of 5 ng/ml) for 48 hours led to marked induction in programmed death, as detected both by DAPI staining of apoptotic bodies (data not shown) and TUNEL, analysed by confocal microscopy (fig 1).

Type I and type II receptor proteins in normal and transformed colon mucosa

Following histopathological and clinical characterisation, the 15 different human colon adenocarcinomas were divided into subgroups according to their TNM classification and degree of dedifferentiation (G1, G2, G3). Five tumours were classified as T2 (G2), six as T3 (G2), two as T3 (G3), and two tumours as T4 (G3). From the paraffin blocks of the individual tumours, 5 μ m slices were cut for receptor immunostaining using the scattered portions of colon mucosa surrounding the neoplasia as the internal reference. Figure 2 shows a representative immunostaining of TGF- β 1 receptors in tumour tissue and surrounding normal colon mucosa from one of the 15 cancer patients (reported as CP in table 1). Both type I and type II receptors had a homogenous distribution in normal colon epithelial cells but type II staining was definitely stronger and also extended to the stroma (fig 2A, B). In the adenocarcinoma cells from this clinical case however type I receptors were dramatically reduced in number, and only scattered cells exhibited a fine granular positive reaction (fig 2C, E). In contrast, expression of type II receptors in cancer tissue appeared to be similar to that of normal mucosa (fig 2D, F). Immunohistochemical findings of all other patients are summarised in table 1. Reduced positivity for TGF- β 1-RI versus the internal control was evident in tumour samples from seven of 15 patients, independent of the degree of tumour cell growth and differentiation. In contrast, only three cases of all tumours examined showed a low content of type II receptor (TGF- β 1-RII).

Lipid peroxidation markers in human colon adenocarcinoma

Steady state levels of free MDA, the most commonly employed index of membrane lipid peroxidation, showed a decreasing trend in tumours compared with the surrounding area (normal colon tissue) in T2 tumours, and a statistically significant reduction in T3 tumours, while in the highly dedifferentiated and advanced T4 tumours the amount of tissue

Table 2 Malonaldehyde (MDA) content and aldehyde-protein adducts in homogenates of biopsy tissues from patients affected by colon adenocarcinoma at different stages of malignancy

Tumour stage	MDA content (nmol/mg protein)	MDA-protein adducts (AFU/mg protein) (390/460 nm ex/em)	HNE-protein adducts (AFU/mg protein) (355/460 nm ex/em)
Normal tissue (15)	0.67 (0.26)	23 (4.0)	30 (5.0)
T2 (5)	0.52 (0.44)	21 (4.0)	23 (3.4)*
T3 (8)	0.29 (0.17)**	16 (5.0)**	21 (2.4)**
T4 (2)	0.64 (0.17)	24 (7.0)	27 (4.5)

Values are mean (SD) (the number of specimens evaluated is indicated in parentheses).

Stage of tumour malignancy was evaluated following the TNM classification.

Significantly different from normal tissue: * $p < 0.05$, ** $p < 0.005$.

Protein-aldehyde adducts were determined as fluorescence exhibited by interaction between protein amino functions and malonaldehyde (MDA) or 4-hydroxynonenal (HNE) at wavelengths of 390/460 nm and 355/460 nm, excitation/emission, respectively.

AFU, arbitrary fluorescence units.

aldehyde was again within the control range. Also, MDA bound to total tissue protein showed a statistically significant decrease only in T3 tumour specimens. Detection of another much more reactive aldehyde stemming from membrane oxidation, HNE, appeared to be a more sensitive marker. In fact,

HNE-protein adducts were found to be significantly reduced in all T2 and T3 tumours (table 2). As in the case of free MDA, the aldehyde-tissue protein adducts detected in the two T4/G3 tumour specimens showed values within the control range.

TGF- β 1 cytokine level in human colon cancer tissue

The actual amount of cytokine present in colon adenocarcinoma tissue at different stages of growth and differentiation was determined by the western technique applied on immunoblotted samples. As an internal positive control, a sample was taken from Crohn's disease mucosa which was characterised by an MDA level of 1.25 nmol/mg protein—that is, about twofold the mean value obtained from normal tissue samples, reported in table 2. As shown by a representative immunoblot in fig 3, the majority of the specimens were examined together in a single gel for TGF- β 1 level. Direct densitometric analysis of the immunoblots was made possible by actual deposition of very similar amounts of the sample's protein, as proved by protein staining with Coomassie blue. In T2 and T3 colon cancers, almost all with G2 differentiation, TGF- β 1 content was without exception dramatically lower than that detected in surrounding normal colon mucosa. In agreement with the corresponding extent of lipid peroxidation, the decrease in TGF- β 1 protein level appeared to be more intense in T3 than in T2 tumours.

As in the case of lipid peroxidation markers, the two T4 tumour samples, both with G3 grading, showed a cytokine level similar to control mucosa while the positive control, Crohn's mucosa, showed a net increase in both TGF- β 1 and lipid oxidation levels.

DISCUSSION

We found that at least with regard to epithelial cells, the cell type from which colon carcinoma originates, TGF- β 1 undoubtedly had a growth inhibitory effect. According to a number of studies, TGF- β 1 exerts its effects through a heteromeric serine/threonine kinase complex involving both TGF- β 1 receptors I and II.^{22–27} Type I receptors apparently require type II receptors to bind the cytokine, and type II receptors require type I receptors for signal transduction.²²

However, the properties of these receptors, in particular type I, have not yet been fully characterised, and a differential role has been suggested for RI and RII proteins in the pleiotropic effects of the cytokine.^{28–29} Indeed, if it were indispensable for TGF- β 1 dependent growth inhibition that receptors I and II be fully expressed and acting together, the proapoptotic effect of TGF- β 1 on CaCo-2 cells reported here would not be possible. Immunochemical analysis of this cell line with rabbit antibodies raised against antihuman RI and RII TGF- β 1 protein receptors, confirmed not to cross react with each other, showed that two thirds of neoplastic cells lack the type I receptor while about two thirds still possess type II receptor.

Figure 3 Western blot analysis of transforming growth factor β 1 (TGF- β 1) protein in patients affected by colon adenocarcinoma at different stages of malignancy. A representative immunoblot. Double protein aliquots (40 μ l each) were taken from biopsy specimens after homogenisation. One aliquot was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue to verify actual protein normalisation (A). The second protein aliquot was also separated by SDS-PAGE but this time processed with a TGF- β 1 polyclonal antibody (B). Densitometric analysis of the cytokine level of the single specimens is reported in terms of arbitrary densitometric units (ADU) (C). Samples: 1, colon mucosa from Crohn's disease patient; 2–4, normal colon tissue; 5–9, mucosa from colon adenocarcinoma at T2 (G2) stage; 10–17, mucosa from colon adenocarcinoma at stage T3 (G2); 18–19, mucosa from colon adenocarcinoma at stage T4 (G3).

A previous study on these two receptors in CaCo-2 cells showed decreased TGF- β 1 binding to type I and especially to type II protein although histochemical analyses were not reported.⁶ Considering the histochemical and functional data together, we may conclude that in this human colon cancer derived cell line, a marked reduction in TGF- β 1 receptors is probably accompanied by significant loss of receptor affinity. Thus the still present susceptibility of CaCo-2 cells to TGF- β 1 dependent induction of nuclear fragmentation and apoptosis appears to be important.

In the light of this finding, the marked decrease in TGF- β 1 protein consistently detectable in T2-T3 (mostly G2) colon cancers is of particular interest: this decrease is presumably a further mechanism of evading cell growth regulation that seems likely to occur in vivo, at least in colon cancer.

The few cases of T4 (G3) tumours recruited do not appear to support this statement as their tissue cytokine levels were within the normal range. However, the high degree of abnormal differentiation of these tumours does not allow correct comparison with less deviated tumours. Of note, transdifferentiation of epithelial cells to mesenchymal cells is one possible event, previously demonstrated in the mammary gland, inducible by TGF- β 1.²⁹ Under this hypothetical condition, mesenchymal-like cells would more efficiently secrete TGF- β 1, which in turn might influence the cell's anomalous differentiation in an autocrine loop.

While not strictly comparable with the corresponding findings achieved in CaCo-2 cells, in samples from human colon adenocarcinoma (15 patients) the distribution of the type I receptor was also more often affected than that of the type II receptor. But, more importantly, the observed changes did not show any relation to the degree of dedifferentiation and neoplastic progression. A previous study in 22 patients with colorectal cancer reported a decrease in TGF- β 1 receptors (especially type I) in only eight cases and in addition found no relation with pTNM staging.¹⁴ In other words, the actual role played by changes in TGF- β 1 receptors, at least in the development of human colon cancer, still remains uncertain. To date, data available do not support this type of change as a general and primary mechanism of selective neoplastic growth.

As far as the mechanism(s) of altered expression of TGF- β 1 in the disease cases under observation is concerned, the fairly constant direct correlation of cytokine levels with those of tissue lipid peroxidation is interesting. Decrease in protein was paralleled in all cases by a low index of lipid oxidation. A similar finding was obtained in our laboratory for TGF- β 1 mRNA and lipid peroxidation in a few cases of human colon cancer.³⁰ In T4 (G3) cancers, both cytokine and lipid peroxidation values were in the normal range. In Crohn's disease, TGF- β 1 protein (this paper) and mRNA,³⁰ as well as lipid peroxidation indices, were consistently found to be increased.

There is a significant body of literature on the association between oxidative stress—that is, the prevalence within the cell of oxidising species over the cellular antioxidant potential—and increased fibrogenic cytokines, in particular TGF- β 1. This association has mainly been found in several disease processes characterised by excessive fibrogenesis, such as atherosclerosis, liver cirrhosis, lung fibrosis, etc (for a comprehensive review see Poli and colleagues^{31–32}). Furthermore, a causative role of oxidative stress (a major event in which peroxidation of cell membrane lipids occurs) in upregulation of TGF- β 1 in fibrotic diseases has been demonstrated both in vitro and in an in vivo experimental system: supplementation of cells or animals with suitable amounts of antioxidant not only prevented lipid peroxidation but also inhibited synthesis of the fibrogenic cytokine and quenched the fibrotic process.^{33–35}

In conclusion, a consistent and marked decrease in the growth inhibitory cytokine TGF- β 1 was found in human colon cancer in early-medium phases of progression while changes

in expression of the specific receptors appeared less frequent and not closely related to progression of the cancer itself. As maintained susceptibility of colon cancer cells to the proapoptotic effect of TGF- β 1 appears to some extent feasible, as suggested by the in vitro experiments with the human CaCo-2 cell line, the net reduction of this cytokine within the tumour mass may be an overall valid mechanism of escape from cell growth regulation. Impairment of membrane lipid oxidation, shown to be associated with tumour progression, would favour this process through reduced availability of aldehyde end products, known to stimulate expression and synthesis of TGF- β 1.

ACKNOWLEDGEMENTS

This research was supported by grants from the Italian Ministry of the University (COFIN 1998, 1999, 2000), the National Research Centre, Rome, Target Project in Biotechnology, the Region of Piedmont, and the two universities involved.

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