Expression of transforming growth factor alpha in experimental gastric carcinogenesis

J I Livingstone, M I Filipe, C Wastell

Abstract

The induction of adenocarcinomas in the glandular stomach of the adult male Wistar rat by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was used as a model to study the expression of the growth promoting peptide, transforming growth factor alpha (TGF α), during experimental gastric carcinogenesis. TGF α was identified using the monoclonal antibody Ab-2 and standard immunohistochemistry, together with a semiquantitative assessment of the intensity of expression. Immunoreactivity was confined to the differentiated compartment of the mucosa while the carcinogen MNNG caused a significant increase in the intensity of TGF α expression ($p < 0.01$), after as little as 16 weeks' exposure. In experimental adenocarcinomas, a change to a previously undescribed pattern of perinuclear TGF α expression was found, which may represent the site of intense TGF α production in the Golgi apparatus after malignant transformation.

(Gut 1994; 35: 604-607)

Transforming growth factor alpha (TGF α) is a 50 amino acid peptide first isolated from fibroblasts transformed by rodent sarcoma viruses,¹ which acts as a ligand for the epidermal growth factor receptor and shares many biological properties with epidermal growth factor.² The distribution of TGF α as determined by monoclonal antibody staining is widespread throughout the gastrointestinal tract but confined to the differentiated compartment of gastric mucosa, suggesting a regulatory role for the peptide in normal gastric epithelium.^{3,4}

TGF α has the ability to induce malignant transformation in some epithelial cells *in vitro*^{5,6} but its role in human carcinogenesis is unknown. In a study of oesophageal diseases, Jankowski showed TGF α immunoreactivity in association with high grade dysplasia in Barrett's oesophagitis⁷ while Yonemura showed that gastric cancers immunopositive for TGF α and epidermal growth factor receptor were associated with advanced disease and poor prognosis.⁸

The experimental induction of adenocarcinoma in the glandular stomach of the rat by the nitroso compound N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) is a well established model of gastric carcinogenesis.⁹⁻¹³ The progression of changes seen during exposure to the carcinogen includes atrophy, regenerative hyperplasia, and dysplasia, which are histologically similar to premalignant stages of human gastric carcinogenesis^{14,15} although intestinal metaplasia is only rarely seen in the rat.¹⁶

The aim of this investigation was to use the

controlled situation of the animal model to study the progression of immunoreactivity of TGF α during the course of experimental gastric carcinogenesis.

Animals and methods

The animals used were adult male Wistar rats of initial weight between 150-200 g. Ten animals were used as controls to study TGF α expression in normal gastric mucosa. A further 40 animals were fed MNNG (Sigma, UK) in the drinking water at a concentration of 150 micrograms/litre for the duration of the experiments, replenished from a stock solution on alternate days and kept in foil wrapped bottles to prevent degradation by light. From 16 weeks of exposure onwards, three animals were killed every six weeks. All remaining animals were killed by 60 weeks.

After removal, the stomachs were cut into longitudinal strips, fixed in formalin, and embedded in paraffin wax. Four micron sections were stained with haematoxylin and eosin and examined for pathological changes; a diagnosis of adenocarcinoma was made only if a lesion showed appropriate histological features and fulfilled Stewart's criteria for the diagnosis of experimental malignancy.¹⁷

Immunohistochemistry was performed using the mouse monoclonal antibody Ab-2 (Oncogene Science, Cambridge, UK), which is specific for human and rat TGF α with no cross reactivity to epidermal growth factor. Sections were pre-digested with trypsin for 15 minutes to expose the antigenic sites before incubation with Ab-2 at a dilution of 5:100, overnight at 4°C. After washing with TRIS buffered saline, rabbit anti-mouse serum (Dako, UK) and streptavidin peroxidase complex (Dako) were added at dilutions of 1:300 and 1:400 respectively, both for 30 minutes, before application of diaminobenzidine and counter staining with Mayer's haematoxylin. After dehydration in alcohol, sections were cleared in xylene, and mounted in diphthalate xylene. A positive control section was included in each batch to ensure consistency of staining. Two types of negative controls were used; in the first, the primary antibody was replaced by TRIS buffered saline. In the second, specific controls were performed by preincubation of the sections with an excess of the TGF α peptide PF008 (Oncogene Science).

The intensity of TGF α immunoreactivity was graded 0-III using the semiquantitative method described by Jankowski.⁷ This assessment was made at the top, neck, and base of the crypts, in both proximal and distal glandular mucosa, unless destroyed by tumour growth. The cellular pattern of staining was also noted. The results were validated independently by a second

The Academic Surgical Unit, Chelsea and Westminster Hospital, London
J I Livingstone
C Wastell

Department of Histopathology, UMDS, Guy's Hospital, London
M I Filipe

Correspondence to:
Mr J I Livingstone, Academic Surgical Unit, Chelsea and Westminster Hospital, 369 Fulham Road, London SW10 9NH.

Accepted for publication
13 August 1993

Characteristics of experimentally induced adenocarcinomas

Tumour	Borrmann type	Differentiation	Special features
1	I	Well	Localised, cystic
2	III	Moderate	
3	I	Moderate	In situ and invasive
4	I	Moderate	
5	I	Poor	Focal tumour
6	IV	Well	Part calcified
7	IV	Well	Part calcified
8	I	Moderate	
9	I	Moderate	
10	II	Poor	Ulcerated
11	I	Well	
12	IV	Moderate	
13	IV	Well	
14	I	Well	Cystic degeneration
15	I	Well	

observer and statistical comparisons between groups were performed using the Mann-Whitney U test for non-parametric data.

Results

Of the 40 animals exposed to the carcinogen MNNG, 15 developed adenocarcinomas, the earliest detected after 26 weeks. The Table summarises the characteristics of the induced tumours. Mucosal atrophy and microcyst formation were seen from the earliest animals killed, progressing to increasing cellular dysplasia.

In control animals, immunopositivity to TGFα was ubiquitous in all the stomachs examined and was abolished in both specific and non-specific negative controls. There was close agreement in interpretation between the two observers. Cellular staining was equally distributed between membrane and cytoplasm with no nuclear staining seen.

The mucosal distribution of TGFα immunoreactivity was concentrated in the differentiated compartment from mid crypt towards the lumen. This was particularly so in proximal, cardiac type mucosa; distal, antral mucosa showed a more even pattern over the lengths of the crypts (Fig 1). Parietal cells showed particularly noticeable staining.

In animals exposed to the carcinogen MNNG where tumour growth had not destroyed the mucosa, a significant increase in the overall intensity of mucosal TGFα positivity occurred compared with normal controls (means (medians) 2.2 (2.0), 1.7 (2.0) respectively, p=0.0094, Mann-Whitney U) (Fig 2). This trend was apparent in both proximal and distal mucosa, but was more noticeable in the first than in the second. The increased TGFα expression was seen in the earliest animals killed.

Premalignant lesions within the mucosa were specifically examined for their pattern of TGFα immunostaining. Overexpression of the peptide compared with the surrounding mucosa was occasionally seen in association with areas of severe dysplasia or microcysts but this was not a uniform finding and a definite trend in these lesions could not be identified.

In tumour tissue, a complete change in the pattern of TGFα immunoreactivity occurred. The previously seen somewhat diffuse cytoplasmic and cell membrane staining was lost but immunopositivity instead became localised to a

Figure 1: Expression of transforming growth factor α in normal proximal glandular mucosa of the Wistar rat. (Above) original magnification ×40 (note concentration towards outer, differentiated compartment); (below) ×250 (note diffuse cytoplasmic staining).

crescent adjacent to the nucleus, usually on the luminal aspect of the nucleus (Fig 3). ‘Peri-nuclear’ immunopositivity was ubiquitous in every experimentally induced cancer in this study but was not seen in non-cancer tissue, whether MNNG exposed or not.

The perinuclear staining pattern was dis-

Figure 2: Intensity of transforming growth factor α immunopositivity in relation to position in crypt for proximal and distal mucosa in (A) normal control animals (n=10) and (B) N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) exposed animals where mucosa not destroyed by tumour (n=25). (See text for statistical comparisons.)

Figure 3: Perinuclear expression of transforming growth factor α in glandular elements of experimentally induced adenocarcinomas. (Above) original magnification $\times 96$; (below) $\times 250$.

tributed among the glandular elements of tumours rather than the stromal elements, the cellular appearance remaining similar whatever the overall grade of tumour differentiation. Only one example of significant carcinoma in situ was found; perinuclear staining was occasionally seen within this lesion but was less noticeable than in overtly invasive neoplasia, most of the lesion showing the more diffuse type of cytoplasmic staining.

The change in the pattern of immunostaining accompanying malignant transformation precluded direct comparison of the intensity of TGF α expression in tumour and non-tumour tissue.

Discussion

The use of MNNG once more proved a reliable method of adenocarcinoma induction in the glandular stomach of the rat, with a series of recognisable intermediate changes.

The distribution of TGF α in normal mucosa was compatible with that described by Thomas³ and Nasim⁴ and is further evidence in support of a regulatory role for this peptide in the growth and differentiation of normal mucosa. The localisation of TGF α to the top and neck region in proximal gastric crypts compared with the more even distribution in distal crypts may result from tighter packing of the cells in the upper regions of proximal crypts but, at a functional level, implies a broadening of the zone of differentiation in distal mucosa. This may, in turn, result from slower mucosal turnover in this region.

The overexpression of TGF α during exposure to the carcinogen is compatible with the proposed role for the peptide in regulating mucosal

growth and regeneration although it is difficult to say which is cause and which effect. In vitro studies show that TGF α can stimulate cell proliferation and, in some cell lines, malignant transformation although not human gastric cancer cells.^{5,6} Nevertheless, these studies suggest that overexpression of TGF α is responsible for the response to mucosal damage rather than being merely a paraprophenomenon, and that the system could, therefore, take part in autocrine positive feedback systems as described by Sporn.¹⁸

The increase in TGF α expression as early as 16 weeks after exposure to MNNG shows that the mechanisms behind mucosal regeneration are capable of rapid response. The lack of localised increases in immunopositivity in association with histologically recognisable premalignant lesions suggests that the role of TGF α in rat gastric carcinogenesis is more of a general mucosal response rather than specific transformation.

The change in TGF α expression associated with malignant transformation was unexpected. Although a variety of patterns of TGF α expression are recognised, localisation into perinuclear deposits has not, to our knowledge, previously been reported in published works.

Clearly, a significant change in the role of TGF α occurred in this animal model during malignant transformation. That the perinuclear pattern was not seen in association with recognised premalignant lesions and only partially in association with carcinoma in situ, suggests that the mechanisms underlying it are late events in carcinogenesis. We put forward two possible interpretations of perinuclear staining; the pattern could represent internalised TGF α in perinuclear endosomes after receptor binding or, alternatively, the site of intense TGF α synthesis in the Golgi apparatus. To substantiate the first would require a similar pattern of immunopexpression by epidermal growth factor receptor, which our preliminary (unpublished) data does not support. To substantiate the second would require the finding of TGF α mRNA by in situ hybridisation or extraction of mRNA for a northern analysis. Alternatively, immunoelectron microscopy would help to determine the cellular organelle involved.

In conclusion, this study supports a central role for TGF α in normal mucosal regeneration and in the response to a mutagenic stimulus. There is a fundamental change in the immunopexpression of the peptide during experimental carcinogenesis. Elucidation of the mechanism may contribute significantly to our understanding of the role of TGF α in malignant transformation.

We are grateful to Dr W J Gullick of the ICRF molecular oncology group for advice on the interpretation of the immunohistochemistry.

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