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Transforming Growth Factors and Related Peptides in Gastrointestinal Neoplasia

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Abstract Transforming growth factor α and β_1 (TGFα and TGF β_1) are representative members of two distinct and expanding families of polypeptide growth factors. TGFα is an epithelial cell mitogen, whereas TGFβ_1 inhibits epithelial cell growth; the role of these factors in contributing to the transformed phenotype is uncertain. Steady state mRNA expression for these growth factors and their receptors in a panel of human colon cancers and adjacent normal mucosa is presented. Based in part on results from transgenic mice in which TGFα is selectively overproduced in the mammary gland, a possible role for TGFα as a tumor promoter in the process of transformation is discussed.

Key words: amphiregulin, colorectal cancer, transforming growth factor α, transforming growth factor β

Based in part on our *in vitro* work with human and mouse keratinocytes, we have proposed a model for the role of transforming growth factors α and β (TGFα and TGFβ) in epithelial cell growth [1]. Both TGFα and TGFβ are produced by nontransformed epithelial cells. Like EGF, TGFα stimulates epithelial cell growth, whereas TGFβ, when activated, inhibits epithelial cell growth. Active TGFβ will override the stimulatory effects of TGFα. We submit that the net effect of these stimulatory and inhibitory pathways may modulate the growth state of nontransformed epithelial cells. It is hypothesized that during the complex process of neoplastic transformation, these growth regulatory pathways become perturbed such that there may be an excess of the stimulatory arm or a defect in the inhibitory signal transduction pathway. We submit that these observations may have relevance to the biology of colonic neoplasia. In the present report, we will discuss recent developments in the study of TGFs, present data on the expression of growth-related genes in a battery of human colon carcinomas and adjacent normal mucosa, and finally speculate on the role of TGFα in the pathogenesis of transformation. The selective nature of this report is emphasized (reviews are referenced in the appropriate sections) and greater attention is given to TGFα than TGFβ.

TGFβ FAMILY OF PEPTIDES

A systematic review of the TGFβ family of peptides is beyond the scope of the present report [2,3]. In mammals, three proteins, frequently referred to as TGFβ isoforms, have been designated TGFβ1, 2 and 3. The genes for these isoforms have been localized to human chromosomes 19, 1 and 14, respectively. In the rat small intestine, the predominant immunostaining for all three proteins is found in the more differentiated villous compartment (J Barnard, pers. comm.). All three closely-related TGFβ molecules are first synthesized as larger precursor molecules that are processed to yield 12.5 kDa mature monomers. Among the family members, the precursor sequences diverge, whereas the mature peptides retain a high degree of homology. The TGFβs are re-

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leased in an inactive form by virtually all cells and tissues; activation is achieved *in vitro* by non-physiological extremes of pH and selected proteases (plasmin, cathepesin D). Two groups recently have cloned one of the three TGFβ binding sites [4,5]; with the identification of the activin A receptor as a serine threonine kinase [6], one may anticipate progress in isolating the elusive type I and type II receptors. The TGFβs exhibit a multiplicity of similar biological actions [2,3]. Chief among these is potent inhibition of epithelial cell growth, although they mediate a number of non-growth-related actions (angiogenesis, immune suppression, matrix induction).

TGFα FAMILY OF PEPTIDES

A comprehensive review of EGF and TGFα has been published recently [7]. It is recognized that TGFα is expressed by many normal cells and tissues; in fact, its range of expression *in vivo* appears far wider than that for EGF [8-11]. Production of TGFα and its receptor (the EGF receptor {EGFr}) is highest in the villous compartment of the rat jejunum [12]. There is an expanding number of members of the TGFα/EGF family of ligands (Fig. 1). In addition to TGFα and EGF, cripto [13,14], heparin-binding EGF-like growth factor (HB-EGF) [15] and amphiregulin (AR) [16-19] have been described. A Schwannoma-derived growth factor (SDGF) has been isolated from the conditioned medium of a rat JS1 Schwann cell line [20]; however, SDGF is reportedly rat AR. These peptides share structural similarities, including the conservation of 6 cysteines of the EGF motif, which in EGF are involved in the 3 disulfide bonds defining the tertiary structure and conferring the ability to bind to the EGFr. Additional EGFr family members include erbB-2 [21-23] and erbB-3 [24,25].

Ciardiello *et al.* recently have reported patterns of expression for TGFα, AR, cripto and erbB-3 in normal human colonic mucosa and colorectal cancer, as well as in hepatic metastasis [26]. AR and cripto mRNA expression was more consistently increased in malignant tissues than adjacent normal tissues, when compared to TGFα mRNA expression. We have also reported comparative mRNA expression for TGFα and AR in psoriasis, as well as in normal and malignant gastric and colonic tissues [27]. In this study, AR expression was more abundant in psoriatic skin than uninvolved skin and normal skin; the relative increase in AR expression appeared more pronounced than previously reported for TGFα in psoriatic skin [28]. In addition, AR expression was consistently higher in gastrointestinal tumors than in adjacent normal mucosa.

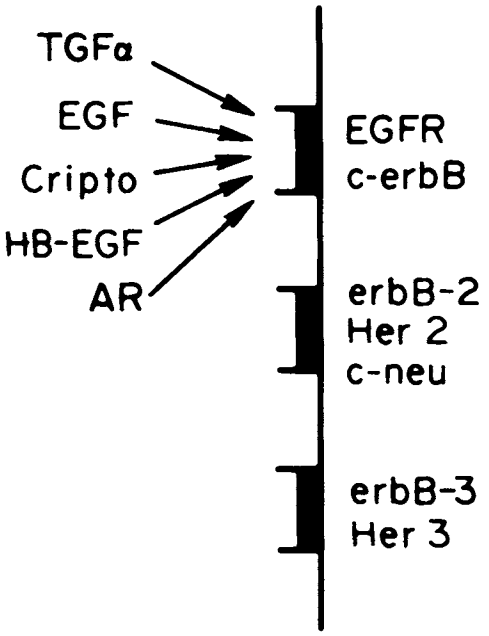

Fig. 1. TGFα/EGF family of ligands and receptors.

SELECTIVE ANALYSIS OF GROWTH-RELATED GENE EXPRESSION IN HUMAN COLON CANCER

We have examined steady state mRNA expression of selected growth-related genes in a battery of human colon cancers and adjacent normal mucosa. The clinical characteristics of the 15 patients examined are presented in Table 1. In the Northern blots presented in Figure 2A and B, 5 μgs of poly (A) RNA was loaded in each lane with equivalent loading and transfer within each pair assured by the constitutively expressed probe β-actin.

In the TGFα family of ligands and receptors, we examined expression of TGFα, EGF, AR, EGFr and c-*neu*. TGFβ1 and TGFβ2 expression was examined but neither the TGFβ3 probe nor any of the TGFβ receptor

probes were available at the time of this analysis. Expression of c-*myc* was also examined. Positive controls for each of the probes are shown in the far right lane.

For a number of reasons, only tentative conclusions can be drawn from these results. The number of patients is small. Although care was taken to obtain a viable piece of tumor at the periphery of the lesion (avoiding the necrotic center), tumors comprise a heterogeneous mix of tissue types (malignant and nonmalignant), and by Northern blot analysis one cannot be certain which cell type(s) is responsible for expression of a certain gene. Ideally, the Northern blot analysis should be complemented by *in situ* hybridization and immunohistochemistry. One would also like to assess the functional status of the receptors [29]. The adjacent mucosa (normal at the gross and light microscopic levels) may not be truly normal, and may represent a field effect.

Notwithstanding these caveats, certain comments seem warranted. EGF was not expressed in any of the tissues, normal or neoplastic (data not shown). AR mRNA expression was a more discriminant marker of cancer than TGFα; the AR transcript was increased in the tumor over normal mucosa in 6 of the 8 paired samples, whereas the TGFα signal was increased in the tumor in only 4 of the 15 pairs. There was no clear correlation between expression of EGFr and c-*neu* in normal vs. malignant tissue. Of interest, TGFβ1 and TGFβ2 expression was increased in, respectively, 6 and 12 of the 15 tumors relative to adjacent normal mucosa. This underscores the view that TGFβ may play a permissive role in tumor progression through its immunosuppressive properties and other indirect actions [30]; these actions may circumvent its role as an epithelial cell growth inhibitor. The most consistent finding of the study was the upregulation of c-*myc* expression in all 15 tumors as compared to adjacent normal tissue. There were no significant correlations between any of the clinical characteristics and patterns of gene expression.

TGFα IN NEOPLASIA

There has been conflicting *in vitro* data as to the role of TGFα in neoplasia. Transfection of TGFα cDNA constructs into non-transformed Rat-1 fibroblasts resulted in a transformed phenotype that was reversed by the addition of antibodies to TGFα [31]. However, NIH 3T3 cells transfected with similar TGFα constructs did not exhibit features of transformation [32]. To better understand the role of TGFα in the development of neoplasia, three groups have examined the consequences of overproduction of TGFα in mice bearing a TGFα cDNA transgene under control of mouse mammary tumor virus (MMTV), metallothionein (MT) and elastase promoters [33-35]. Mammary carcinoma was observed in postlactational female mice under control of the MMTV and MT promoter. In the report of Jhappan *et al.* [34], hepatic neoplasia also occurred in MT-TGFα mice and was more frequent than mammary carcinoma. In the MT-TGFα transgenic mice [34, 35], breast cancer developed despite low expression of the transgene in the mammary gland relative to other tissues (liver, kidney, gut). Of interest, Sandgren *et al.* [35] observed colonic hyperplasia within 2 months of zinc administration to MT-TGFα mice, but to date no colonic carcinomas have been reported in these mice. These findings suggest that mammary epithelium is particularly susceptible to development of neoplasia in the setting of enhanced TGFα production and that in this *in vivo* model TGFα acts as an oncogene. How does enhanced production of TGFα lead to mammary neoplasia? Clearly TGFα is a mitogen for mammary epithelium. Proliferation of normal mammary epithelial cell lines is stimulated by TGFα *in vitro* [36,37] and local application of TGFα in slow-release form to mammary glands of 5-week-old mice is associated with local alveolar and ductal growth [38]. *In vivo*, DNA synthesis is enhanced in the mammary glands of MT-TGFα transgenic mice [34]. However, overproduction of TGFα by itself appears insufficient to cause mammary neoplasia and additional events are necessary [33]. One such event may be upregulation of EGFr. Recent *in vitro* transfection studies indicate that marked upregulation of TGFα production may contribute to neoplasia if sufficient numbers of EGFr are present, and it has been suggested that an abundance of both ligand and its cognate receptor are required to achieve a critical threshold in terms of the mitogenic signal cascade to induce a malignant phenotype [39]. In human tumors, overexpression of the EGFr has been reported in mammary carcinoma and squamous cell carcinoma of the head and

TABLE I. Clinical characteristics of 15 patients, whose colonic tissues were examined in Figure 2. Abbreviations: mod. = moderately; diff. = differentiated; m. = muscularis; mets. = metastasis.

	AGE	SEX	SITE	SIZE (cm)	GRADE	LEVEL	NODES	METS.
1	65	male	R colon	9 X 5.5	mod. well diff.	into m. propria	17/17 (-)	(-)
2	40	male	R colon	5 X 5	poorly diff.	through serosa	8/17 (+)	(-)
3	40	male	R colon	4 X 4	mod. diff.	deeply into m. propria	1/18 (+)	(-)
4	61	male	R colon	3 X 5	mod. to poorly diff.	into serosa	1/14 (+)	(+)
5	67	male	R colon	6 X 3	mod. diff.	into m. propria	2/11 (+)	(-)
6	75	male	R colon	3.5 X 3.5	mod. diff.	into m. propria	1/3 (+)	(-)
7	60	male	sigmoid	3 X 3	mod. well diff.	through serosa	9/14 (+)	(-)
8	75	female	sigmoid	5 X 5	mod. diff.	through serosa	7/14 (+)	(-)
9	52	male	sigmoid	4 X 6	mod. well diff.	through m. propria	2/5 (+)	(-)
10	39	male	sigmoid	3.2 X 3	mod. well diff.	through m. propria	4/14 (+)	(-)
11	72	female	rectum	2.5 X 3.5	mod. well diff.	into m. propria	8/8 (-)	(-)
12	56	male	rectum	4 X 3.5	mod. diff.	into m. propria	7/12 (+)	(-)
13	65	female	rectum	4 X 3.5	mod. diff.	through m. propria	1/11 (+)	(-)
14	78	female	rectum	8 X 5	mod. diff.	into perirectal soft tissue	8/9 (+)	(+)
15	63	male	rectum	7 X 4	mucinous with mod. diff.	into perirectal soft tissue	4/10 (+)	(-)

neck [40,41]. In this context, we have shown that mammary tissues harboring histologic abnormalities express high levels of the TGFα transgene and display increased expression of the endogenous EGFr mRNA [33].

This still does not provide a mechanism by which overproduction of TGFα leads to neoplasia. In the next section, we advance the hypothesis that TGFα, when sufficiently overproduced, may act as a tumor promoter.

EGF/TGFα AS TUMOR PROMOTERS

Carcinogenesis is a complex, multistep process that has been divided into multiple discernible stages, including initiation, promotion and progression; in these likely overlapping stages, environmental and endogenous factors act through a variety of different biochemical and genetic mechanisms [for review see 42, 43]. *In vivo* studies in mice have provided circumstantial evidence that EGF contributes to mammary and colonic neoplasia. In a strain of mice with a high incidence of spontaneous mammary carcinoma (C3H), sialoadenectomized virgin females had a nearly 5-fold decrease in the incidence of mammary tumors at 52 weeks, compared to sham operated controls (12.8% vs 62.5%) [44]. Sialoadenectomy is thought to mediate its effects by depleting the mouse of a major source of EGF. Administration of EGF at the time of sialoadenectomy increased the tumor incidence to 33%. In a subsequent report, sialoadenectomized virgin females of strains also predisposed to mammary cancer had a reduced incidence of premalignant histological changes [45]. 1,2-Dimethylhydrazine (DMH) is recognized to cause colonic carcinoma in rodents. It is a procarcinogen that becomes oxidized to its active alkylating metabolite, methylazoxymethanol (AOM). The tumors that are induced tend to occur in the distal colon and rectum, a pattern of distribution which is similar to human colon cancer. Both adenocarcinoma and squamous cell carcinoma of the anal canal develop. In one DMH study in mice [46], intermittent administration of EGF resulted in a 3-fold increase in squamous cell cancer of the anus (although there was no significant increase in adenocarcinoma). These observations, coupled with additional *in vitro* data, support a role for EGF as a tumor promoter.

Tumor promoters can be defined as compounds which have very weak or no carcinogenic activity when tested alone but enhance tumor formation when applied repeatedly following a low or suboptimal dose of a carcinogen (initiator) [42]. Most promoters induce proliferation in target cells, yet a number of agents which induce proliferation in specific target tissues are not active as promoters. While the precise mechanisms of action for all promoters are not understood, the likeliest common action of these agents is to cause a selective clonal expansion of the initiated cell population resulting in a clinically evident premalignant lesion and increasing the number of cells at risk for further changes in neoplastic progression [42]. A classic tumor promoter is 12-*O*-

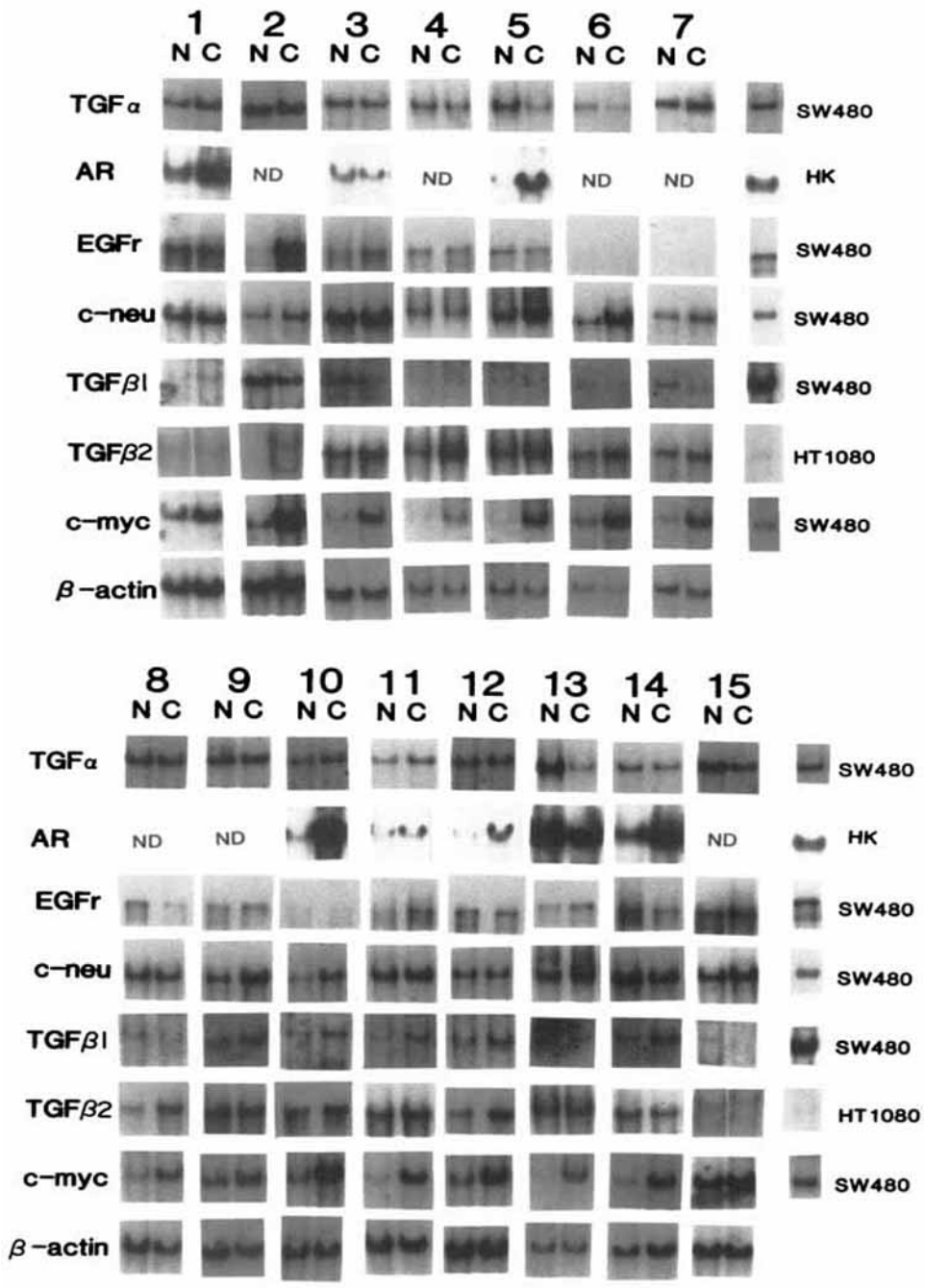

Fig. 2. Total cellular RNA from adjacent normal mucosa (N) and colon cancer (C) in 15 patients was prepared by homogenization in 4 M guanidine isothiocynate and pelleted through a cesium chloride cushion by centrifugation. Poly (A) RNA was isolated with oligo (dT) cellulose, and 5 mg of poly (A) RNA/lane was separated by electrophoresis in 1% agarose/formaldehyde gels. RNA was transferred to nitrocellulose paper and these filters were hybridized to ^{32}P-labelled cRNA probes for TGFα, AR, EGFr, TGFβ2 and c-neu and cDNA probes for EGF, TGFβ1, c-myc and b-actin. Hybridization and post-hybridization washes were as described previously (11,52,53).

tetradecanoylphorbol-13-acetate (TPA). The discovery that TPA binds and activates the enzyme protein kinase C (PKC) has revealed common molecular mechanisms for growth factor activity, signal transduction and activation of specific cancer genes (oncogenes) [43].

In this context, TPA and EGF mediate similar effects. Both agents activate PKC and downregulate the EGFr (although reportedly through different mechanisms; reviewed in 47). Administration of both agents *in vivo* results in epidermal hyperplasia [48; M Stahlman, pers comm]. Based on the biochemical and functional homology between EGF and TGFα, we have demonstrated that selected effects of TPA may be mediated through enhanced production of TGFα. Administration of TPA to cultured keratinocytes results in a 20-fold induction of TGFα mRNA and protein [47]. Furthermore, intracolonic administration of a colonic tumor promoter deoxycholate (DOC) to rats results in a time and dose-dependent increase in colonic TGFα production [49; RJ Coffey, unpub observ]. The effects of TPA and DOC are pleiotropic, but these similar findings have led to the hypothesis that TGFα, when sufficiently overproduced, may act as a tumor promoter.

We have explored this hypothesis in MMTV-TGFα transgenic mice [33,50]. The transgene is expressed in the small ducts and alveoli of the mammary gland at approximately 5 weeks of age. Spontaneous mammary tumors do not occur before 320 days in line 29 virgin females. A single initiating dose of 7,12-dimethylbenzanthracene (DMBA) (0.5 mg via intragastric instillation) was administered to line 29 virgin transgenic females (TG) and their nontransgenic littermates (NTG) at 8 weeks of age. To date, 8/12 TGs and 0/15 NTGs have developed mammary carcinoma by 220 days of age; median age at tumor formation in TGs was 120 days [51].

A minimalist interpretation of these data is that there is a significant interaction between DMBA and TGFα. Since the initiator is given after onset of TGFα expression, these data do not exclude that TGFα, through it mitogenic effect, may act to expand the population of cells upon which the initiator acts, as well as to expand the initiated population of cells through its classic tumor promoting effect. We are presently administering DMBA at 3 weeks (prior to the onset of TGFα expression). If the timing of tumor formation is similar in the two groups, this would provide circumstantial evidence that TGFα is acting predominantly as a tumor promoter. If tumors form earlier in the former group, both actions cited above may be operative. In this regard, it will be of interest to examine TGFα production in premalignant lesions of the colon.

SUMMARY

We have reviewed recent developments in the study of TGFs with emphasis on the role of TGFα and related peptides in neoplasia. A challenge will be to understand the redundancy of these growth factors and to determine distinct localization and/or functions for individual members in normal physiology and their possible participation in malignant transformation. Colonic epithelium, with its recognized transition states from normal mucosa to polyp to cancer, offers a useful model system to conduct such studies.

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