Oral Oncol, Eur J Cancer, Vol. 29B, No. 1, pp. 3–7, 1993
Printed In Great Britain

0964–1955/93 $6.00 + 0.00
Pergamon Press Ltd

TGF-α and Oral Carcinogenesis

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Transforming growth factor-alpha (TGF-α) has been shown to be consistently expressed by tumours of epithelial origin, particularly squamous and renal carcinomas. Epithelial tumours are often found to concurrently express the receptor to TGF-α, namely epidermal growth factor receptor (EGFR), at elevated levels. The simultaneous expression of TGF-α and EGFR by the carcinoma cells is thought to trigger the autocrine growth pathway, resulting in uncontrolled proliferation. Similar observations of elevated TGF-α/EGFR expression have been detected in oral squamous carcinomas from human and animal sources. By RNA blotting analyses, elevated levels of TGF-α/EGFR expression have been consistently observed with malignant human and hamster oral cancers. Interestingly, by use of cellular localisation techniques of *in situ* hybridisation and immunohistochemistry, we have shown that there is another, previously unnoticed, cellular source of TGF-α at oral tumour sites. Eosinophils are a major cellular source of this growth factor in oral cancer and their presence is tightly associated with malignant oral epithelium. Furthermore, transformed oral epithelium *in vivo* has been shown to be associated with elevated levels of EGFR expression. Thus quantitative changes in TGF-α and EGFR levels in the microenvironment of oral tumours have been observed *in vivo*. With the hamster oral cancer model, the stage is therefore set to elucidate the cellular and molecular contributions of TGF-α and EGFR in the process of oral cancer development.
Oral Oncol, Eur J Cancer, Vol. 29B, No. 1, pp. 3–7, 1993.

INTRODUCTION

TRANSFORMING GROWTH factor-alpha (TGF-α) was discovered 14 years ago as an activity, together with TGF-β, that can reversibly transform normal rat kidney cells in culture [1]. It is a member of a growth factor family of which epidermal growth factor (EGF) was the first to be discovered [2]. Other members of this family include amphiregulin [3], heparin-binding EGF-like growth factor [4], and three members of the poxvirus family: vaccinia virus growth factor (VVGF) [5, 6], myxomavirus growth factor (MGF) [7], and Shope fibroma growth factor (SFGF) [8]. These molecules all have a core sequence containing six characteristically spaced cysteines that participate in the formation of three intracellular disulphide bonds. This core sequence, together with the disulphide bond configuration, constitutes the molecular basis whereby these peptide growth factors interact with the same cellular receptor, named epidermal growth factor receptor (EGFR) since EGF was first found to interact with it.

TGF-α is encoded by an mRNA that is 4.5–5.5 kb long [9–11]. The 50-amino acid mature TGF-α peptide is contained within a precursor of 159 (rat and hamster) [10, 11] or 160 (human) [9] amino acids. This precursor TGF-α sequence consists of a signal peptide (22 amino acids), a pro-TGF-α sequence (16 or 17-amino acids), the mature TGF-α peptide (50 amino acids), a transmembrane hydrophobic sequence (23 amino acids), and the cytoplasmic domain (39 amino acids) (Fig. 1). The cleavage recognition sequence (Ala–Val–Val) is located at both the N- and C-termini of the mature TGF-α peptide sequence. This suggests that specific proteases with elastase-like properties are involved in the release of the mature form of TGF-α from the extracellular portion of the TGF-α precursor. It has recently been shown that activation of the protein kinase C pathway by the tumour promoter 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) enhances this posttranslational cleavage [12].

Besides the secreted 50-amino acid mature peptide form, TGF-α can present itself in multiple precursor forms [13]. Many TGF-α synthesising cells do not necessarily secrete the soluble form [14, 15], but invariably they all have cell surface TGF-α precursor molecules [12, 16, 17]. These data together suggest that the transmembrane cell surface TGF-α, in addition to the soluble form, is also a normal cellular form of the cytokine [12, 15]. Cell surface transmembrane TGF-α has been shown to interact with EGFR on adjacent cells [15, 18], resulting in cellular proliferation [19]. This type of cell–cell interaction can result in very specific and localised "juxtacrine" stimulation of EGFR bearing cells.

EXPRESSION OF TGF-α IN NORMAL CELLS AND INVOLVEMENT IN PRENATAL DEVELOPMENT

TGF-α mediates its biological activities through the same receptor as for epidermal growth factor (EGF), namely the EGF-receptor (EGFR). Most mammalian cells, with the exception of a few differentiated white blood cells, do express EGFR [20, 21]. Thus most mammalian cell types are potential targets of TGF-α/EGF. Initially thought to be expressed only in transformed tissues, TGF-α has since been shown to be expressed by normal human skin keratinocytes [22, 23], bovine anterior pituitary cells [24], rat maternal decidua [25], mouse blastocysts [26], and activated macrophages [27, 28]. It is currently thought that under normal conditions epithelial

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Received 4 Aug. 1992; accepted 13 Aug. 1992.

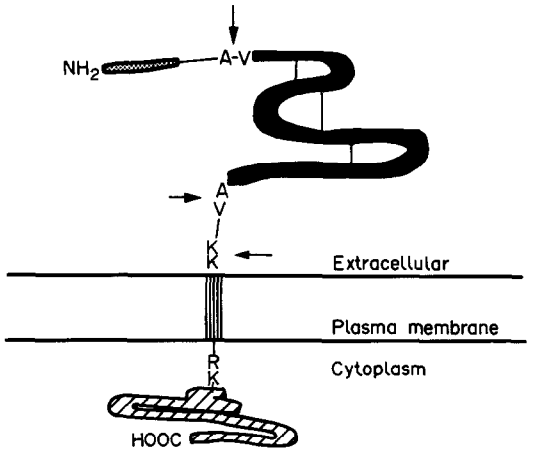

Fig. 1. Schematic representation of the TGF-α precursor as a transmembrane molecule. The amino terminal signal peptide (grey) is followed by the pro-TGF-α sequence and then by the 50 amino acids TGF-α mature peptide with six conserved cysteines. The transmembrane domain (striped bars) anchors the molecule into the plasma membrane. This is followed by the cytoplasmic domain (cross hatch). Arrows indicate the proteolytic cleavage sites. A = alanine, V = valine, R = arginine, K = lysine.

cells are the major producers of TGF-α [13]. Besides keratinocytes, activated macrophages are found to be an alternative cellular source of TGF-α at cancer sites and healing wounds. We have recently shown a novel cellular source of TGF-α associated with the processes of wound healing and carcinogenesis. The granulocytic leucocytes, eosinophils, are capable of expressing high cellular levels of TGF-α [29–31]. Our studies in oral carcinogenesis and animal wound healing revealed that eosinophils infiltrate prominently into the tissue sites in a time dependent fashion. The infiltrated eosinophils, not macrophages, were the major cellular source of TGF-α detected by *in situ* hybridisation and immunohistochemistry [31, 32].

Like EGF, TGF-α is a pleuripotential peptide. Though initially thought to be a transformation-associated protein, it is now clear that TGF-α is expressed by a large variety of normal cell types. These recent findings have led to the suggestions that besides being implicated in various pathological processes, TGF-α is functional in normal development and physiology of cells. It is conceivable that in view of the increasing list of normal adult cell types shown to express TGF-α, compared to the limited cellular sources of EGF, TGF-α may eventually turn out to be the major normal physiological ligand of EGFR. This possibility is further reflected in three recent reports demonstrating the presence of TGF-α in human saliva [33], plasma [34], and milk [35].

TGF-α IN CARCINOGENESIS

The linkage of TGF-α to malignant transformation was implied since its initial discovery in the culture medium of fibroblasts transformed by sarcoma viruses [1]. Originally named as "sarcoma growth factor", it was later renamed after it was discovered that it had the ability to reversibly induce normal rat kidney (NRK) cells to form colonies in soft agar [36], a phenotype most closely correlated with malignant transformation [37]. It was discovered during the purification of this activity that the transformation activity was due to the cooperation of two structurally unrelated proteins, TGF-α and TGF-β [38]. The fact that TGF-α was first discovered in transformed but not in normal fibroblasts and that it can cooperate with TGF-β to transform NRK cells led to the belief that TGF-α can importantly contribute to malignant transformation.

These findings have led to searches for human tumours that express TGF-α [13, 14]. Examination of a panel of human tumour cell lines and tumour biopsies has revealed that TGF-α expression is a common finding [13]. Notably, all squamous and renal carcinomas were found to express high levels of TGF-α mRNA [14] and concurrently expressing high levels of EGFR mRNA. This led to the formulation of the TGF-α/EGFR autocrine hypothesis in epithelial carcinogenesis [39]. Namely, transformed cells can acquire a growth advantage or autonomy by virtue of their ability to secrete a growth factor to which they respond, resulting in unlimited self-stimulation [40].

However, it was soon discovered that most normal keratinocytes, which bear EGFR, also express TGF-α [22, 23]. The finding that TGF-α expression is not restricted to tumour keratinocytes challenges the autocrine pathway in the genesis of epithelial tumours. In support of the idea that normal expression of TGF-α/EGFR is a natural circuitry of cellular physiological response, a number of studies have demonstrated expression of high levels of TGF-α in mammalian cells but only a few resulted in malignant transformation. Expression of TGF-α in early-passage NIH 3T3 cells [41], cultured primary keratinocytes [42], and skin papillomas [42] resulted only in conferring growth advantages upon the transfected cells and did not result in malignant transformation. On the other hand, expression of TGF-α in immortalised cell lines such as NRK fibroblasts [43], Rat-1 fibroblasts [44], the NOG-8 mouse mammary epithelial cells [45], and established NIH 3T3 cells [46] did result in malignant transformation. These data suggest that TGF-α is oncogenic only in cell types that have already traversed far along the multistep process of carcinogenesis [13].

An important contribution to further the understanding of the role of TGF-α and EGFR in carcinogenesis is the demonstration that quantitative and/or qualitative differences between normal and tumour cell expression of TGF-α and EGFR might account for the normal regulation of epithelial proliferation on one hand versus the uncontrolled growth of tumour epithelium on the other. It was the elegant studies of Di Fiore *et al.* [47] and Di Marco *et al.* [48] that demonstrated that there is a minimum quantitative requirement for both TGF-α and EGFR in order to result in malignant transformation of NIH-3T3 cells in culture. These results suggest that a high level of TGF-α expression, together with EGFR upregulation, commonly seen in squamous and renal carcinomas, can importantly contribute to the malignant transformation process. However, it is important to note that while elevated levels of EGFR expression have frequently been noted in epithelial tumours [14], it is not yet known with certainty if the transformation of keratinocytes is accompanied by an increase in TGF-α expression. We have recently quantified the level of TGF-α mRNA expression by *in situ* hybridisation in hamster oral epithelia during oral cancer development induced by 7,12-dimethylbenzanthracene (DMBA) [49]. We found that concomitant with the onset of epithelial hyperplasia and thereafter (dysplasia and carcinomatous), the cellular level of TGF-α mRNA in the transformed basaloid keratinocytes only demonstrated a moderate increase by about 2-fold [49].

Whether this moderate increase in transformed keratinocyte-derived TGF-α is sufficient to drive the malignant transformation of EGFR overexpressing epithelial cells remains to be experimentally tested.

In order to evaluate whether the cellular levels of TGF-α and/or EGFR expression can influence the carcinogenesis process *in vivo*, transgenic animals overexpressing either one or both of these components have been generated. Five studies have reported the biological consequences of overexpressing TGF-α in transgenic mice [50–54]. Chronic overexpression of TGF-α *in vivo* was found to be directed to multiple tissues throughout the life of the transgenic mouse. The following consistent findings were detected: epithelial hyperplasia, pancreatic metaplasia, adenocarcinoma of the breast, and liver neoplasia [50–52]. Matsui *et al.* [51] showed that in transgenic mammary epithelium, besides elevated TGF-α expression, there was concomitant elevated EGFR expression. Thus *in vivo*, the localised overexpression of TGF-α can lead to increased epithelial proliferation and in certain tissues can lead to tumour development, particularly when EGFR expression is concurrently up-regulated. Whether these localised tissue conditions in the transgenics resulting in TGF-α overexpression (by the epithelial tissues) and EGFR up-regulation (presumably also in the epithelial elements) are reproduced in non-transgenic tumour-bearing animals is presently not clear. There is as yet no *in vivo* demonstration that the transformation of keratinocytes is accompanied by simultaneous increase of TGF-α and/or EGFR synthesis.

Merlino *et al.* reported the creation of transgenic mice that overexpress EGFR under the control of a chicken β-actin promoter [55]. Careful examination of one of the transgenic mouse lines (AE24) revealed that overexpression of EGFR was only detected in the testis, leading to sperm flagellar axonemal disruption and male sterility. No increase in tumour incidence was reported. It would be of interest to create transgenic mouse lines that would target the EGFR gene to epidermal cells [54] leading to keratinocyte overexpression of EGFR and evaluate the incidence of spontaneous tumour development, in relation to specific TGF-α stimulation.

TGF-α IN ORAL CARCINOGENESIS

Our laboratory is engaged in investigations to elucidate the role of TGF-α and EGFR in oral carcinogenesis. The animal oral cancer model, the Syrian hamster cheek pouch, is well suited for the study of oral carcinogenesis [56–58]. Our work began with the finding that the expression of the cellular protooncogene c-*erb*B1 is elevated in hamster cheek pouches that were transformed by the carcinogenic chemical 7,12-dimethylbenzanthracene (DMBA) [59, 60]. Since the cellular homologue of c-*erb*B1 is the EGFR [61], this prompted us to search for the possible aberrant expression of either EGF and/or TGF-α by the chemically transformed hamster oral keratinocytes. Examination of tumour-bearing hamster cheek pouches has shown that TGF-α, not EGF, was consistently expressed [62]. Human oral tumours, either freshly obtained or propagated in culture, were subsequently shown to similarly express both TGF-α and EGFR mRNAs by northern blot analysis [63]. The concurrent expression of TGF-α and EGFR mRNAs by these oral tumours led to the hypothesis that the autocrine mechanism was operative in the genesis of oral cancers. A number of other studies have since implicated the participation of cellular protooncogene c-*erb*B1 and/or EGFR in oral carcinogenesis [64–73].

One of the many advantages of the hamster cheek pouch oral cancer model is that mucosal alterations resembling that seen in human oral biopsy specimens (hyperplasia, dysplasia, and carcinoma) can be similarly produced. Since it was thought that the tumour-associated TGF-α originates primarily from the transformed oral mucosa, we set out to plot the onset of TGF-α expression by the hamster oral mucosa during the chemical transformation process. These studies led to two unexpected findings. First, normal as well as transformed (hyperplastic, dysplastic, and carcinomatous) oral mucosa was detected to contain TGF-α mRNA by *in situ* hybridisation [49]. Transformed oral epithelium has approximately twice the amount of TGF-α mRNA as normal oral mucosa. Second, the major cellular source of TGF-α at tumour developing sites was found to be not from the transformed oral epithelium but instead from the eosinophils, as part of the host's inflammatory response infiltrating into the tumour [29]. Eosinophils were detected to be the major cellular source of TGF-α mRNA synthesis while immuno-reactive TGF-α was demonstrated in these leucocytes by immunocytochemistry. We have subsequently shown that human eosinophils can also express TGF-α [30], and are the major cellular source of TGF-α in human oral [74] and colon carcinomas [30].

The discovery that eosinophils are the major cellular source of TGF-α at oral tumour sites was a surprise finding. Despite a number of clinical reports that have demonstrated the association of tissue eosinophilia and tumour [75–81], including head and neck cancers [75], their role in carcinogenesis remains unclear. Using the hamster oral cancer model, we have shown that eosinophils infiltrate progressively and prominently during the tumour development process, tissue eosinophilia is associated with malignant oral epithelium, and that the majority of eosinophils associated with malignant oral epithelium are expressing TGF-α mRNA [32].

In view of the current body of information, TGF-α is likely to be a normal mitogenic cytokine in oral epithelium. Together with EGFR, TGR-α is normally involved in the controlled autocrine growth control of oral keratinocytes. The keratinocytes are the major cellular source of TGF-α in normal oral mucosa. The cellular levels and/or forms of TGF-α and EGFR in normal oral keratinocytes are unlikely to result in malignant transformation. The role of TGF-α and EGFR in transformation of oral mucosal tissues remain to be experimentally elucidated. However it is clear that perturbations to the expression of both TGF-α and EGFR, qualitatively and/or quantitatively, can result in malignant transformation. Dissection of the molecular and cellular apparatus at oral tumour development sites will eventually lead to a better understanding of the involvement of this cytokine/receptor pathway in oral carcinogenesis. Situations where there is upregulation of EGFR and enhanced TGF-α expression at the same site will create a microenvironment that will favour malignant transformation of the affected oral epithelial cells. It is therefore important to determine the biological scenarios that might encourage the progression of carcinogenesis through the TGF-α and EGFR pathway. The moderate elevation of TGF-α by transformed oral epithelium is unlikely to result in necessary quantities of TGF-α to interact with oral keratinocytes that are expressing elevated levels of EGFR. The challenge is therefore to define the cellular and molecular signals and requirements that can lead to altered TGF-α and EGFR expression at sites of oral cancer development.

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Acknowledgements—I am most grateful to the following people in my laboratory who have contributed to the work in oral carcinogenesis. They include Allen L. Chang, Tao Chiang, Phoebe Chow, Ming Yung Chou, Aram Elovic, Robert Gertz, Mohammad Ghiabi, Jim McBride, Karekine Matossian, Christian Meyer, Naoyoshi Nagura, Bai-Zheng Song, Randy Todd, Takanori Tsuji, Diane Trickler, Lisa Tyler, and James Yang. I am most fortunate to have collaborative inputs from Stephen J. Galli and Peter F. Weller of the Harvard Medical School, and R. Bruce Donoff and George T. Gallagher of Harvard School of Dental Medicine. I am immensely thankful to the support and encouragement from G. Shklar, my mentor, who inspired me to the field of experimental oral carcinogenesis.—Supported by Public Health Service Grant DE 08680. The author is the recipient of a Research Career Development Award from National Institute of Dental Research (DE 00318), and a Cancer Research Scholar Award from the American Cancer Society.