Asynchronous Modulation of Transforming Growth Factor α and Epidermal Growth Factor Receptor Protein Expression in Progression of Premalignant Lesions to Head and Neck Squamous Cell Carcinoma

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ABSTRACT

The development of head and neck squamous cell carcinoma occurs as a result of the accumulation of genotypic and phenotypic alterations in the upper aerodigestive tract mucosa. Up-regulation of epidermal growth factor receptor (EGFR) and its ligand, transforming growth factor α (TGF-α), have been identified previously as early events in head and neck carcinogenesis. To determine the timing of increased TGF-α and EGFR protein expression in the development of head and neck cancer, we examined progressive mucosal dysplasias from three distinct and complimentary patient groups: (a) samples from patients with lesions demonstrating different degrees of dysplasia (n = 22) compared with mucosa samples from gender and age-matched controls (n = 8); (b) patients with lesions demonstrating different degrees of dysplasia at a single time point (n = 3); and (c) patients who progressed over several years to invasive cancer at the site of dysplasia (n = 7). Immunohistochemical analysis with monoclonal antibodies specific for TGF-α and EGFR were used to detect protein expression in all specimens. Protein levels were further quantitated using a computerized image analysis system. In all three groups, we found that TGF-α protein levels were elevated in mild dysplasia compared with control normal mucosa and were not further modulated with increasing degrees of dysplasia. In contrast, EGFR levels were relatively low in mild dysplasia and increased with higher degrees of dysplasia. These findings indicate that up-regulation of TGF-α and EGFR are distinct events both chronologically and, possibly, mechanistically in the pathogenesis of head and neck squamous cell carcinoma.

INTRODUCTION

Head and neck squamous cell carcinoma is an epithelial malignancy arising in the mucosa of the upper aerodigestive tract. Potential anatomical sites affected include the oral cavity, oropharynx, hypopharynx, and larynx. Patients often present with multiple tumors involving different sites (synchronous) or, more commonly, develop second primary tumors following curative treatment of the index tumor (metachronous). The frequency of multiple primary tumors underscores the "condemned" nature of the entire mucosa reinforcing the "field cancerization" hypothesis initially proposed by Slaughter et al. (1) in the 1950s. The development of an invasive cancer is thought to arise from step-wise progression through increasing degrees of mucosal dysplasia, which are often asymptomatic. The identification of genetic alterations in these premalignant lesions and their timing would enhance our understanding of the etiology of squamous epithelial carcinogenesis as well as provide a much needed opportunity to target patients at risk and prevent the formation of the initial and/or second primary tumor.

Genetic alterations that precede phenotypic changes are likely to represent early events in cancer development. We have demonstrated previously elevated TGF-α and EGFR mRNA and protein in the histologically normal mucosa several centimeters away from the primary tumor site in patients with head and neck cancer compared with levels in control normal mucosa from patients without cancer (2, 3). We have also shown that TGF-α and EGFR protein expression levels in the tumor can be accurately quantitated by immunohistochemical staining with monoclonal antibodies followed by computerized image analysis of the staining (3). In that report, protein levels quantitated using this technique were comparable to mRNA expression levels in the same tissue samples. Here, we examined the timing of elevated TGF-α and EGFR protein expression in progressive degrees of mucosal dysplasia from three distinct and complimentary groups of patient samples: (a) dysplastic mucosa specimens compared with control mucosa from patients without cancer; (b) patients with lesions demonstrating different degrees of dysplasia at a single time point; and (c) patients with progressive dysplasia and subsequently carcinoma at a single site over the course of several years. The results demonstrate in all

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3 The abbreviations used are: TGF, transforming growth factor; EGFR, epidermal growth factor receptor; MOD, mean absorbance.
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against 17 amino acids in the COOH-terminal portion of the tissue. Mucosal dysplasia was defined as alteration of the patients undergoing resection of their lesions were obtained.

MATERIALS AND METHODS

Tissue Samples and Patients. Archival tissue samples (paraffin-embedded) of head and neck mucosal dysplasias from patients undergoing resection of their lesions were obtained from the diagnostic histopathology laboratories at the University of Pittsburgh Medical Center. Normal oropharyngeal mucosal samples were obtained from age (±5 years) and gender-matched control patients undergoing nononcological procedures (e.g., uvulopalatopharyngoplasty).

Pathological and Immunohistological Examination of Tissue. Mucosal dysplasia was defined as alteration of the epithelial cells with respect to size, shape, polarity, and organization, where thickening of the basal layer was accompanied by disorderly maturation of the suprabasal cells. The degree of mucosal dysplasia was determined by the severity of epithelial cell involvement and was accordingly subdivided into mild, moderate, and severe grades. Staining was performed on tissue sections using monoclonal antibodies specific for TGF-α (Ab2; Oncogene Science) and EGFR (Genosys/Cambridge Research) as described previously (3). The TGF-α antibody was raised against 17 amino acids in the COOH-terminal portion of the molecule (34–50), and the EGFR antibody recognizes the extracellular domain, which is not affected by ligand-binding and corresponds to residues 580–591 of human EGFR. Positive controls consisted of normal skin known to have abundant TGF-α or a squamous cell carcinoma with elevated EGFR expression (4). Negative controls consisted of replacement of the primary antibodies with a matched murine IgG subclass antibody.

Computerized Image Analysis of TGF-α and EGFR Staining. The mean labeling concentration of the immunohistochemical staining was evaluated under ×40 on a SAMBA 4000 Image Analysis System (Image Products International, Chantilly, VA) as described previously (3). Background staining is accounted for by the computer program by analyzing several sections of each slide. The first background reading consists of an empty field on the glass slide, and the second reading includes nonepithelial tissue on the slide. The computer then automatically subtracts the background from each reading. By separating and deleting stromal areas, only mucosa expression was included in the computer analysis. Staining heterogeneity was measured by concentration heterogeneity, which represents the concentration variation coefficient between cells and structures and is equal to the ratio of concentration SD:mean concentration. In our dysplastic samples, concentration heterogeneity of TGF-α staining ranged from 0.19 to 0.42 with a mean of 0.3 (SD, 0.065). Concentration heterogeneity of EGFR staining of the dysplasias ranged from 0.22 to 0.47 with a mean of 0.33 (SD, 0.071). The concentration heterogeneity of the normal controls was also low (mean TGF-α, 0.34; mean EGFR, 0.28), which suggests that any staining heterogeneity of TGF-α or EGFR was not specific to dysplasias. The mean labeling concentration is the mean of the mean absorbances measured over the labeled areas within the structures (in the case of cytoplasmic or membrane immuno-enzymatic labeling). Twelve representative sections of each sample were analyzed, and the result was reported as the MOD of the 12 values.

Statistical Analysis. Unless otherwise indicated, Student’s t test was used to compare the MOD between groups. A two-sided alternative hypothesis was used to determine P, and a result of <0.05 was considered significant.

RESULTS

EGFR and TGF-α Levels Are Elevated in Dysplasia Compared with Control Normal Mucosa: EGFR, but not TGF-α, Levels Are Increased with Progressive Dysplasia. We first examined TGF-α and EGFR protein staining characteristics in samples of mucosal dysplasia obtained from 22 patients at a single time point and compared these to samples of normal mucosa from 8 patients without dysplasia matched for age (±5 years) and gender. There were 10 samples of mild dysplasia, 7 samples with moderate dysplasia, and 5 specimens demonstrating severe dysplasia. In all 22 cases, we found that
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TGF-α staining intensity was elevated in mild dysplasia compared with control normal mucosa and did not appear to be further modulated by increasing cellular atypia. In contrast, EGFR staining increased with progressive degrees of dysplasia (Fig. 1).

To facilitate statistical analysis of these results, levels of TGF-α and EGFR protein expression were quantitated in the 22 samples of dysplastic lesions and 8 normal controls using the SAMBA 4000 Cell Image Analysis System (Fig. 2). The mean MOD of TGF-α protein levels in each group of dysplastic samples was greater than in the normal mucosa from control patients without cancer ($P = 0.001$). There was no difference in TGF-α expression levels detected in mild dysplasias compared with levels in moderate or severe dysplasias. By contrast, EGFR...
levels in moderate and severe dysplasia were elevated compared to control normal mucosa \((P = 0.001)\), and each was elevated when compared to mild dysplasia \((P = 0.004)\). There was no statistical difference in EGFR protein levels between groups demonstrating moderate and severe dysplasia. These results suggested that up-regulation of TGF-\(\alpha\) and EGFR are asynchronous events in the molecular pathogenetic pathway leading from normal mucosa to carcinoma. At a mean follow-up of 52 months (range, 32–69 months), one patient with moderate dysplasia and one patient who presented with severe dysplasia had developed invasive carcinoma at the site of prior dysplasia at 3 and 4 years, respectively. Both patients demonstrated high EGFR (and TGF-\(\alpha\)) expression levels in their initial dysplasia. To determine whether there was a difference between expression of TGF-\(\alpha\) and EGFR in the basal versus the suprabasal epithelium, layer-by-layer computerized image analysis was performed. For both TGF-\(\alpha\) and EGFR expression, there was no significant difference between mean expression levels (MODs) in basal epithelial cells compared with suprabasal epithelial cells (data not shown).

**EGFR, but not TGF-\(\alpha\), Is Increased in Lesions with Higher Degrees of Dysplasia Presenting at a Single Time Point in Individual Patients.** To determine whether the increase in TGF-\(\alpha\) and EGFR protein levels were indeed due to the degree of histological atypia and were not attributable to some other characteristics of the upper aerodigestive tract mucosa of individual patients, we examined three patients who presented at a single time point with several premalignant lesions, representing the spectrum of dysplasias. In all three cases, TGF-\(\alpha\) protein staining intensity was elevated in the mildly dysplastic lesion as well as the moderately and severely dysplastic specimens. In contrast, although EGFR protein was detected in mild dysplasia, it was further increased with progressive degrees of cellular atypia (Fig. 3). These results demonstrate that similar to findings in lesions from multiple patients (Figs. 1 and 2), TGF-\(\alpha\) is up-regulated early without further elevation, whereas EGFR protein levels continued to increase with progressive degrees of cellular atypia in a single patient with multiple lesions displaying the spectrum of dysplasia. Thus, the temporal sequence of TGF-\(\alpha\) and EGFR up-regulation is clearly linked to the degree of atypia and cannot be attributed to some other feature(s) of the upper aerodigestive tract mucosa of individual patients.

**EGFR, but not TGF-\(\alpha\), Increases with Progressive Dysplasia and Subsequent Cancer Formation at a Single Site.** Although most patients who present with preneoplastic mucosal lesions do not progress to invasive carcinoma at the site of the dysplasia, we identified seven patients who ultimately developed squamous cell carcinoma at the same upper aerodigestive mucosal site where dysplasia had been identified previously. TGF-\(\alpha\) and EGFR protein expression were examined in these specimens to determine the level and timing of TGF-\(\alpha\) and EGFR up-regulation in dysplastic lesions documented to have progressed to carcinoma. In all seven cases, there was elevation of TGF-\(\alpha\) protein in mild dysplasia at the time of initial presentation. Despite the progression of cellular atypia in the lesion over time, TGF-\(\alpha\) protein levels were not further increased. EGFR, however, was present in mild dysplasia at presentation, and the intensity of staining increased with progressive mucosal dysplasia (Fig. 4). To perform statistical analyses of these results, levels of TGF-\(\alpha\) and EGFR protein expression were measured in 8 samples of mild dysplasia and 11 samples of moderate and severe dysplasia using computerized image analysis (Fig. 5). No difference was detected in TGF-\(\alpha\) expression levels in mild dysplasias compared with levels in moderate and severe dysplasia. However, EGFR levels were elevated in moderate or severe dysplasias compared with levels in mild dysplasias from the same patients \((P = 0.001)\). These data demonstrate that in a premalignant lesion that progresses to invasive cancer, the timing of TGF-\(\alpha\) and EGFR up-regulation relative to the degree of dysplasia is the same as that observed in the first two groups. TGF-\(\alpha\) protein levels are elevated early and are not further modulated by increasing degrees of cellular atypia, whereas EGFR levels may be further elevated with progressive dysplasia. The findings in this last group indicate that the timing of up-regulation of TGF-\(\alpha\) and EGFR holds for the most relevant group of dysplastic lesions, which will ultimately result in carcinoma.

**DISCUSSION**

Immunostaining of dysplastic mucosa from patients prior to the development of head and neck squamous cell carcinoma revealed TGF-\(\alpha\) and EGFR protein expression in the epithelium of all samples. The level of protein expression, quantitated with the aid of computerized image analysis, demonstrated that although TGF-\(\alpha\) protein levels were increased in mild dysplasia compared with control normal mucosa, they were not further modulated by increasing degrees of cellular atypia. In contrast, EGFR protein expression levels increased with progressive dysplasia. By examining TGF-\(\alpha\) and EGFR expression in several
dysplastic lesions from a single patient, we determined that TGF-α and EGFR protein levels are linked to the degree of dysplasia and not to some other feature(s) of the aerodigestive mucosa within individual patients. Our finding of elevated TGF-α and progressive increase of EGFR expression in dysplastic lesions at a single site where there was subsequent tumor formation indicate that this temporal sequence holds for the most critical subset of lesions that are destined to progress to carcinoma.

Members of the EGFR family have been implicated in several human carcinomas, including head and neck cancer. We and others have found that this increase in EGFR expression is accompanied by an elevation in TGF-α production, providing indirect evidence of an autocrine growth pathway (2, 5). Modulation of EGFR using several strategies, including antisense oligonucleotides and monoclonal antibodies, as well as inhibition of EGFR kinase activity with specific inhibitors, resulted in inhibition of head and neck cancer cell growth (6). Recent studies in our laboratory have demonstrated that growth of head and neck squamous cell carcinoma cell lines, but not normal mucosal epithelial cells, can be inhibited by down-modulation of TGF-α using antisense oligonucleotides, indicating that TGF-α and EGFR are participating in an autocrine growth pathway in head and neck squamous cell carcinoma.4 We and others have reported previously the detection of EGFR with increasing degrees of dysplasia, generally within the same specimen as the tumor (3, 7, 8). When elevation of a growth factor and/or its receptor is detected in dysplastic tissues at the time of cancer presentation, the precise sequence of events leading to malignant transformation is difficult to determine. Laryngeal biopsies, which showed a higher proportion of epithelial cells that stained positively for EGFR, were more likely to progress to invasive cancer over time when compared with lesions that demonstrated lower EGFR expression levels (9). Elevated TGF-α protein expression has been reported in verrucous leukoplakia compared with control normal mucosa; however, levels of TGF-α expressed in the dysplasias were similar to those detected in squamous cell carcinomas (10). TGF-α expression levels in leukoplakia can be down-modulated by systemic treatment with retinoic acid (11). By examining TGF-α and EGFR expression levels in progressively dysplastic lesions that culminated in invasive carcinoma, we were able to more precisely place up-regulation of TGF-α and EGFR into a chronological sequence in this tumor system.

Not all lesions found in the upper aerodigestive tract mucosa are truly premalignant. For example, the vast majority (~80%) of leukoplakias show no evidence of cellular atypia upon histological examination. It is further estimated that only 5% of patients who present with leukoplakia will eventually develop cancer (12). In the larynx, approximately one-third of dysplastic lesions will progress to cancer, whereas ~9% of patients whose lesions show no evidence of dysplasia will develop carcinoma (13). The association between genetic alterations and cancer formation is incompletely understood. In oral dysplasia, approximately 14% will progress to invasive cancer over the course of 20 years (14); however, using chromosomal in situ hybridization, more than 50% of oral premalignant lesions show evidence of genetic alterations (15). Like squamous cell carcinoma, epidemiological studies have shown that the risk of oral dysplasia is associated with tobacco and alcohol exposure and declines following smoking cessation (16). The availability of a readily determined biological marker would greatly enhance our ability to identify patients at high risk for subsequent tumor formation and thus more effectively prevent this frequently fatal malignancy.

EGFR expression levels in the premalignant lesion appear to be a sensitive factor in predicting the neoplastic potential of dysplastic tissues. This suggests that EGFR may serve as a biological marker to identify high-risk subgroups and guide prophylactic therapy. Such therapy could theoretically include monoclonal antibodies against EGFR, such as those used in patients with lung squamous cell carcinoma (17, 18) or fusion proteins or immunotoxins against TGF-α or EGFR using toxins elaborated by Pseudomonas or Diphteria species (19–21).

Retinoids have been shown to be effective both in eradicating premalignant head and neck lesions and as chemopreventive agents in head and neck squamous cell carcinoma (22). Our previous finding of modulation of EGFR gene transcription by retinoic acid in head and neck cancer cells provides a biologically plausible explanation for the clinically observed effects of retinoids (23) and reinforces the notion that EGFR levels may serve as an intermediate end point in chemoprevention trials.

REFERENCES

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