Induction of Transforming Growth Factor β-1 in Cervical Intraepithelial Neoplasia in Vivo after Treatment with β-Carotene

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Abstract

Transforming growth factor (TGF) β1 is a potent growth inhibitor of epithelial cells. Loss of responsiveness to TGF-β1 and/or loss of TGF-β1 itself may be important in the progression of cervical intraepithelial neoplasia to invasive cervical cancer.

Retinoids have antiproliferative effects on epithelial cells and have been used as chemopreventive and chemotherapeutic agents for several human cancers. There is evidence that retinoids exert their effects by promoting the induction of TGF-β. The aim of this study was to determine whether the expression of TGF-β1 was altered in patients enrolled in a clinical trial designed to test the therapeutic efficacy of β-carotene, a carotenoid metabolized to retinol, in cervical intraepithelial neoplasia. Using an immunohistochemical technique, tissues were stained with two types of antisera that react with the intracellular and extracellular forms of TGF-β1. Matched cervical biopsies taken from 10 patients before and after treatment with β-carotene were immunostained simultaneously to allow direct comparison of relative staining intensity.

A significant increase in intracellular TGF-β1 immunoreactivity was noted in cervical epithelial cells in patients with cervical intraepithelial neoplasia after treatment with β-carotene (P = 0.003). These results demonstrate regulation of a TGF-β isoform in vivo in humans in response to β-carotene administered as a chemopreventive agent.

Introduction

It is generally accepted that invasive cervical carcinoma is the end result of a continuum of precursor lesions clinically classified as CIN2 grades I–III (1, 2) and that HPV infection of the cervix is an important cofactor in the development of CIN and invasive cancer (3).

Retinoids, structural and functional analogues of vitamin A, exert profound effects on the differentiation and proliferation of cervical epithelial cells. Vitamin A deficiency has been shown to induce metaplastic changes in mouse cervical epithelium (4). In addition, studies have found significantly increased risks of cervical dysplasia/carcinoma in situ and invasive cancer in women with low serum or cervicovaginal cellular levels of β-carotene, which is derived from plant sources and converted to retinol (5, 6). Finally, novel therapeutic approaches using retinoids in the prevention and treatment of preinvasive (CIN) lesions (7) and invasive carcinoma of the cervix (8, 9) in humans have shown some effectiveness. These experimental and clinical observations suggest that the maintenance of normal cervical epithelial morphology and function may depend on the availability of retinoids and that abnormalities of cellular proteins that mediate their effects may be important determinants of cervical neoplastic transformation.

TGF-β is an inhibitory growth factor that has potent antiproliferative effects on a number of epithelial cell types (10). In cell culture, TGF-β inhibits proliferation of normal genital squamous epithelial cells and induces expression of its own mRNA, suggesting that it may function as an autocrine regulator of cell growth and gene expression (11–13). Retinoids, which are natural and synthetic derivatives of vitamin A, have been shown to induce the expression of TGF-β (14) and to alter expression of its receptors in vitro (15).

Our group has recently completed a Phase III clinical trial designed to evaluate the efficacy of β-carotene, a potent dietary source of vitamin A, in promoting stabilization or regression of cervical intraepithelial neoplasia II. The purpose of this study was to examine whether the expression of the epithelial growth inhibitory peptide TGF-β1 was modulated in vivo by β-carotene administered as a chemopreventive agent.

Materials and Methods

Patients and Tissue Specimens. Paraffin-embedded cervical biopsy specimens were obtained from 10 women who participated in a Phase III clinical trial designed to evaluate the efficacy of β-carotene in promoting the stabilization or regression of CIN. Women who participated in this trial had CIN II...
diagnosed by colposcopically directed biopsy and were randomly assigned in a double-blind fashion to a treatment group that received either 30 mg of β-carotene or placebo daily. All subjects were followed at 3-month intervals by colposcopy for 9 months, at which time a colposcopically directed biopsy was obtained. Baseline and 9-month biopsy specimens were examined from 10 patients with low-grade intraepithelial neoplasia who received β-carotene.

**Immunohistochemical Staining and Analysis.** All tissue was fixed in 10% buffered formaldehyde at room temperature for a period of 4–12 h. Tissues were embedded in paraffin, and 5-μm sections were cut and stained with H&E. CIN biopsy specimens stained by H&E were classified by standard criteria as CIN I or CIN II (16).

Tissue sections were deparaffinized, rehydrated, blocked for endogenous peroxidase activity with 1.0% hydrogen peroxide in methanol, and digested with hyaluronidase [1 mg/ml in 0.1 mol/liter sodium acetate buffer (pH 5.5) with 0.85% NaCl for 30 min at 37°C; Sigma, St. Louis, MO]. Nonspecific protein binding was blocked by incubation with 1.5% goat serum in PBS for 20 min. Sections were incubated overnight with either normal rabbit serum as a negative control (0.02 mg/ml), LC antibody, or CC antibody, which correspond to the intra- and extracellular forms of TGF-β1, respectively (0.02 mg/ml). Each antibody type has different epitope specificity that enables it to recognize conformational changes between intracellular and extracellular forms of TGF-β1 (17). The reaction product was then detected with a biotinylated goat anti-rabbit IgG antibody followed by avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). The color reaction was developed with diaminobenzidine (Research Genetics, Huntsville, AL) activated with hydrogen peroxide and counterstained with hematoxylin.

Matched cervical biopsies taken from 10 patients before and after β-carotene treatment were stained simultaneously for the LC and CC antibodies to allow direct comparison of relative staining intensity and to minimize the possibility of experimental variation.

Staining intensity of each antibody was graded from 0 to 3 (0 = negative, 1 = weak staining, 2 = moderate staining, 3 = strong staining) for epithelial and stromal compartments. Differences in epithelial and stromal staining before and after treatment with β-carotene were evaluated using the Wilcoxon matched pairs signed rank test with the Statistical Package for the Social Sciences. Statistical significance was accepted at  P < 0.05.

**Results and Discussion**

Of the 10 patients enrolled in the β-carotene clinical trial that were examined in this study, 6 demonstrated persistent CIN lesions and 4 demonstrated regression to CIN I after 9 months of β-carotene. Six of 10 patients had serum β-carotene levels 10 times greater at the completion of the study compared with initial levels, and all patients had β-carotene levels that at least doubled.3

After treatment with β-carotene, a marked increase in both intra- and extracellular TGF-β1 immunoreactivity was noted in all three epithelial cell layers (Fig. 1, Table 1). The most marked increase was seen in the intracellular parabasal and midepithelial cell layers (P = 0.003). A less pronounced increase was noted in intracellular TGF-β1 in the superficial cell layer after β-carotene therapy (P = 0.008). There was also an increase in immunoreactivity for intracellular TGF-β1 within stromal cells (P = 0.024). No significant increase in extracellular stromal TGF-β1 was noted. Five patients demonstrated staining for intracellular TGF-β1 in the basal cell layer after β-carotene treatment. This was not noted in any of the pretreatment samples studied. There was no difference in the degree of increased immunoreactivity for intra- or extracellular TGF-β1 between lesions that remained stable versus lesions that demonstrated regression. Control staining was negative in all cases.

Previously, our group has found that the expression of TGF-β1 was significantly decreased in the epithelial component of CIN and invasive carcinoma, suggesting that the loss of this peptide may be an important early event in malignant transformation of the uterine cervix (18).

Glick et al. (19) examined the role of TGF-β in tumor progression and malignant conversion by comparing the expression of TGF-β1 and TGF-β2 between low-risk and high-risk mouse skin papillomas. Sixty percent of high-risk papillomas and 91% of squamous carcinomas were TGF-β negative. A strong association was noted between epidermal loss of TGF-β1 and TGF-β2, hyperproliferation, and malignant progression.

TGF-β has been shown to down-regulate the transcription of the E6 and E7 early viral transforming regions of HPV 16 and 18 and inhibit cell proliferation of HPV 16 and HPV 18 DNA containing nontumorigenic genital epithelial cell lines (13, 20). Tumorogenic HPV 16-positive cancer cell lines, however, have shown resistance to the growth-inhibitory effects of TGF-β, suggesting that loss of sensitivity to TGF-β1 growth inhibition may be important in the pathogenesis of cervical neoplasia (13).

Retinoids, anti-estrogens, and synthetic progestins, all of which are ligands for the steroid/thyroid superfamily of nuclear receptors, have been shown to induce the synthesis and secretion of TGF-β (21–23). TGF-β is inhibitory to breast epithelial cells cultured in vitro, and treatment of breast cancer cell lines with tamoxifen results in a rise in TGF-β1 mRNA expression with an associated inhibition of cell growth (23). The synthetic progestin gestodene, which also inhibits the growth of breast cancer cells, has been shown to stimulate a large increase in the secretion of TGF-β1 and TGF-β2 (25).

We have demonstrated, for the first time (MEDLARS 1980-present), evidence consistent with in vivo regulation of a TGF-β1 isoform in humans in response to a chemopreventive agent in women with CIN. In contrast to a similar previous study that demonstrated increased immunohistochemical expression of extracellular stromal TGF-β in 10 breast cancer patients receiving tamoxifen (23), this study demonstrated the most pronounced increase in expression within epithelial cells. Of particular significance is the finding that intracellular TGF-β1 was prominent in the basal cell layer in one-half of the patients studied after treatment with β-carotene. This is in contrast to our previous findings (18), which consistently demonstrated the

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3 P. R. Palan, personal communication.
absence of intracellular TGF-β in the basal epithelial cells of normal and neoplastic cervical tissue.

Mutagenic events that occur during carcinogenesis do not lead to irreversible phenotypic damage until late stages of the carcinogenic process. In general, it has been found that during the early stages of carcinogenesis, epithelial cells retain their sensitivity to the growth inhibitory actions of TGF-β (26). Thus, if neoplastic cells present in low-to-moderate grades of CIN are stimulated to produce and/or retain their responsiveness to TGF-β by a chemopreventive agent, they could theoretically be subject to negative autocrine regulation. In addition, stimulation of TGF-β production in the basal cell layer, as noted in this study, could lead to a decrease in cell proliferation, thus inhibiting the generation of aneuploid clones, some of which could harbor genetic changes necessary for malignancy.

Cancer chemopreventive trials usually use two end points: long-term cancer incidence end points and short-term intermediate biological end points. Ideally, intermediate end points should show differential expression in normal and high-risk tissue. They should be measurable in small tissue specimens and should be quantitative and reproducible, with the degree or pattern correlating with the stage of carcinogenesis, and in the long-term with a reduction of cancer incidence (27). Observations in this preliminary work and previous studies support TGF-β expression as a valid intermediate biological end point in cervical carcinogenesis and add insight to the regulatory activity of carotenoids and retinoids in cervical epithelium.

**Acknowledgments**

We thank Dr. Kathleen C. Flanders for generously providing both the intracellular and the extracellular antibodies for TGF-β1.
References


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