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Nutrient Antioxidants in the Pathogenesis and Prevention of Cervical Dysplasias and Cancer

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Abstract The role of nutritional factors in biochemical interactions that are part of an oncogenic process or inhibit free radical proliferation have attracted considerable interest in relation to molecular mechanism(s) and the natural history of human cancer. Epidemiologic and experimental studies have drawn attention to the association between dietary micronutrient deficiencies and the incidence of neoplastic and malignant lesions. In the last two decades, the role(s) of retinoids, carotenoids, tocopherols and water-soluble antioxidant vitamins, and allegations of anti-tumor properties in the daily dietary consumption of fresh fruits and green leafy vegetables, have captured the attention of an increasingly sensitive diet- and health care-conscious public, the biochemical community, and industrial food producers. Moreover, recent epidemiologic and compelling advances in molecular biology have linked the presence of restricted human papillomavirus (HPV) subtypes to cervical carcinoma and precursor lesions. In the present report, we identify and review measurable effects of dietary deficiencies of selected antioxidant micronutrients (*i.e.*, β-carotene and vitamins A, C, and E) and their associations with known cervix cancer risk factors in the pathogenesis and potential prevention of cervix dysplasias, presumed to be the precursor lesions of cervix cancer.

Key words: Antioxidants, cervix cancer, nutrition, prevention

The uterine cervix of the human female is the ideal organ site for a successful prospective study of nutritional biomarkers potentially useful for screening, diagnosing, monitoring and evaluating the safety and efficacy of promising chemotherapeutic agents with a role in the prevention of cervix cancer. The cervix is anatomically accessible, visible, palpable, and easily biopsied. There is a widely accepted cytomorphologic working hypothesis that the natural history of cervix cancer involves a progression of increasingly severe histopathologic graded lesions. The contributions of molecular biology provide compelling evidence that an oncogenic potential in specific subtypes of human papillomavirus (HPV)-DNA is a primary etiologic factor in cervical carcinoma [1,2]. The biomedical community is now challenged to elucidate the molecular mechanisms of the multifactorial etiology, biology, carcinogenesis, and potential prevention of cervix cancer.

The Pap test is widely available and publicly accepted as a cervix cancer screening mode. Using enhanced magnified visualization, pathologic cervical sites exfoliating abnormal epithelial cells can be directly biopsied at colposcopy, constituting a unique system to investigate the role of diet and nutrition in the natural history of cervix cancer. Women having abnormal Pap smears need to be investigated because they are presumed to be at risk for cervix cancer. With informed consent and a good patient-physician

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orientation, compliance can be achieved from volunteer subjects for extended essential protocol requirements. Frequent Pap smears, colposcopy examinations, and specifically indicated biopsies to clarify unexpected histopathologic progression in individual patients can be part of the safety net in prospective protocols.

Against this background, we initially asked the question: Do women who are exfoliating abnormal dysplastic cervicovaginal epithelial cells or cervix cancer cells seen in Pap smears have a deficiency in dietary vitamin A intake and a metabolic defect in retinol and retinoic acid metabolism that influences the maturation and differentiation of cervical epithelium? From this initial question, we expanded our horizons to include the role of isoprenoid antioxidants and ascorbic acid.

This report reviews accrued evidence, obtained from observational and case-control studies, of the relevance of selected essential antioxidant micronutrient vitamins (retinol, β-carotene, ascorbic acid and α-tocopherol) to the pathogenesis and prevention of cervix cancer.

PATHOGENESIS OF CERVICAL DYSPLASIA AND ITS DIAGNOSIS

The epidemiology of cervix cancer as a venereal disease has been extensively studied and is well known. Early onset of sexual activity, multiple sexual partners, promiscuity, prostitution, smoking, infrequent use of barrier contraception, questionable long-term use of oral contraceptives, and genital tract HPV infections are widely accepted risk factors.

Seeking to clarify the role of this viral sexually transmissible DNA in the etiologically linked oncogenic process of cervix cancer, researchers use molecular hybridization techniques capable of identifying a multitude of subtypes of cervix HPV infections [3–5]. Impressive evidence from laboratories throughout the world has identified HPV types (6, 11, 16, 18, 31) in tissue obtained from cervix cancer and genital and cervical warts [3,4]. HPV 16 and 18 are predominant in cervical and genital carcinomas [3,4], and HPV 16 has been prominently identified in high-grade cervical dysplastic lesions. HPV 16 and 18 DNA sequences are frequently integrated into the genome of cervical cancer cells, whereas they exist as episomal plasmids in most premalignant cervical lesions. The biology of this system is activated by virus propagation. In previous studies, we found 86% of our study population with cervix dysplasias to be infected with diverse types of HPV [5,6].

A concern in recruiting subjects for nutritional studies is use of the Pap test as a definitive recruiting tool; in many published reports, cytology alone is the basis for designating volunteer women subjects as "normal" controls or "abnormal" cases. In the current studies, protocols were designed to monitor and record Pap smear findings, as well as results of colposcopy examinations in the same subject sequentially over extended periods of time. We are concerned that a single baseline Pap smear may bias the interpretations of selected nutrient indices in studies designed to detect dynamic transient nutritional biochemical changes associated with rapid progression in cytomorphologically graded cervix lesions. In this latter connection, the sensitivity and credibility of any single Pap smear and any single colposcopically directed cervical biopsy may be questioned as representing the most definitive histopathologic cervical abnormality in an individual patient. Frequently, transient colposcopic lesions may be observed when Pap smear reports are negative. Such findings confirm problems associated with false negative Pap smears and the biased consequences implicit in evaluating specific assays. When the Pap test detects a cancer cell, it is invaluable. However, other categorical interpretations, including revisions contained in the publicized Bethesda System, are subject to the constraints of technologic or subjective cognitive limitations. Subjective limitations also apply to the visual perception of colposcopic criteria identifying presumptive cellular abnormalities that are subsequently biopsied. Thus, colposcopy is limited and additionally complicated by the colposcopist's need to estimate and biopsy one or more cervical sites perceived to be most abnormal and morphologically most randomly representative of the oncogenic process. In our experience, one-third of colposcopically directed biopsies have been interpreted by staff pathologists to have no significant pathology.

In a 1978 paper, Koss [7] classically reviewed the subjectivity and morphologic limitations that account for differences among individual staff pathologists in their diagnostic interpretations of

cytologic and tissue samples. His experience is widely recognized by others. It is abundantly clear that until the molecular biology of cervix cancer is unequivocally established, cytologic and histopathologic evidence are of limited value in prognosticating whether any individual precursor cervical lesion will biologically progress, remain stable, or spontaneously regress [8–10].

THE NUTRIENT ANTIOXIDANTS

The importance of vitamin A to cancer has been recognized since the 1920s. Wolbach [11] first noted effects upon cellular differentiation in 1925. Epidemiologic and animal studies have drawn attention to the associations between dietary nutrient deficiencies and the incidence of neoplastic and malignant lesions. In the past several decades, the role of retinoids, carotenoids and antioxidant vitamins, along with allegations of antitumor benefits in daily consumption of fresh fruits and vegetables, has captured the attention of industrial food producers, an increasingly sensitive diet- and health care-conscious public, and the biomedical community [12–16]. The function and mechanisms of nutritional constituents in biochemical processes, especially their inhibition of free radical proliferation, have generated considerable interest as to their molecular role in the natural history of some cancers [17–20]. Our multidisciplinary nutritional collaboration has been focused along the following lines.

Dietary Vitamin A and Vitamin C In Cervical Dysplasias

In a case-control study (87 cases and 82 controls) of women receiving a routine Pap test, matched for age, ethnicity, socioeconomic status and parity, nutrient intake was estimated by computer analysis of three-day food records and 24-hour food recall. The subset of those with severe dysplasia or carcinoma *in situ* (CIS) had a total dietary vitamin A intake below the pooled median of 3,450 IU and/or a β-carotene intake below the pooled median of 2,072 IU compared with normal controls ($p < 0.05$ and $p < 0.025$, respectively). Odds ratio revealed an approximately 3-fold greater risk for severe dysplasia or CIS in women with lowered vitamin A or β-carotene intake [21].

In the same dietary survey, the controls in a subset of 49 matched pairs revealed a mean vitamin C intake per day of 107 mg during a three-day food record compared to 80 mg for cases ($p < 0.01$). In analyzing the matched pairs, 29% of the cases had a vitamin C intake less than 50% of the recommended daily allowance compared to 3% of the controls [22].

Intracellular Cervical Binding Proteins for Retinol and Retinoic Acid

Intracellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein (CRABP) have been detected and quantified in blinded analyses of colposcopically directed biopsies obtained from normal women and patients histopathologically diagnosed as having dysplasias or CIS of the cervix who completed the computerized nutrient survey. Both proteins sediment in the 2S region and are of similar molecular weight (M.W. ≈ 14,000). CRBP was not detected in 78.8% of 33 abnormal biopsies compared to 23.5% of 34 normal control tissue specimens ($p < 0.005$) [23].

Plasma Ascorbic Acid, β-Carotene and Retinol Levels in Uterine Dysplasias and Cancer

In a case-control study of 80 women who obtained a Pap test, the mean plasma concentration of ascorbic acid in 34 control subjects having negative cytologic tests, negative colposcopic findings, and no known gynecologic dysfunction was significantly higher (0.75 mg/dl) than the 46 cases who had a mean of 0.36 mg/dl ($p < 0.001$). Women with abnormal findings, in contrast to the control subjects, had statistically significant decreases in their plasma ascorbic acid levels, regardless of the severity of the dysplasia or CIS. Of interest is finding the greatest decrease in women diagnosed with chronic inflammatory cervicitis [24].

Plasma β-carotene levels of coded venous blood samples obtained from 141 women recruited for a cross-sectional study were determined by HPLC. When decoded, 104 patients had cervical dysplasias or cancer, and 37 women were normal volunteer controls recruited with informed consent from family planning clinics. The mean plasma β-carotene levels were signifi-

cantly reduced in all women with dysplasias (n = 72) and in the 32 women with cancer. There was an inverse relationship between plasma β-carotene levels and the histopathologic grade of the dysplasia. The lowest mean plasma β-carotene level of 7.4 μg/dl was detected in the cancer patients. In contrast, all patients in the various groups (control, cervical dysplasias, and cancer) had normal plasma retinol levels ranging from 61.7 to 64.8 μg/dl [25].

Plasma Levels of β-Carotene and α-Tocopherol in Cervix Dysplasias and Cancer

In a cross-sectional study, the plasma levels of β-carotene, retinol and α-tocopherol were simultaneously determined by HPLC on coded peripheral venous blood samples obtained from 116 women. When decoded, 36 women had negative Pap smears, normal colposcopy, were not using oral contraceptives, and were considered normal controls. Eighty women had colposcopically directed biopsies and tissue samples histopathologically interpreted as grades of dysplasia; ten women had cervix cancer.

Significantly reduced plasma levels of β-carotene and α-tocopherol were observed in women with dysplasias and cancer ($p < 0.001$ and $p < 0.005$, respectively). In groups with advanced dysplasias, the percentage of smokers was markedly increased and the women were comparatively older ($p < 0.001$). A strong association was noted between plasma β-carotene and smoking, independent of the cervical pathology. However, this was not evident with respect to α-tocopherol. These findings suggest interdependent biologic activity between β-carotene and α-tocopherol in the pathogenesis of dysplasias and cervix cancer [26].

Effects of Smoking and Oral Contraception on Plasma Ascorbic Acid and β-Carotene Levels in Healthy Women

Significant decreases in both reduced and total plasma ascorbic acid levels were observed in normal healthy women who were smokers. Age was an interacting variable. Oral contraception, barrier or IUD methods, or smoking had no effect on plasma ascorbic acid among women less than 26 years old, but decreases in both reduced and total ascorbic acid were evident in smokers 26 years or older. Total exposure to smoking, *i.e.* pack-years, appears to be a significant confounding variable when interpreting plasma ascorbic acid levels [27].

A cross-sectional study of plasma β-carotene levels determined by HPLC in 149 healthy women was carried out on coded peripheral venous blood samples. Of these volunteer women, 88 were oral contraceptive users and 61 were not. Among those using oral contraceptives, 21 were smokers; there were 18 smokers among the women not using oral contraceptives. Oral contraceptive users had significantly reduced plasma β-carotene levels ($p < 0.001$) and higher plasma retinol levels ($p < 0.001$). There were no differences in plasma β-carotene or retinol levels among users of IUDs or barrier methods. Cigarette smoking alone was associated with significantly reduced plasma β-carotene levels in nonusers ($p < 0.001$). Combined cigarette smoking and oral contraceptive usage were associated with low plasma levels; the results appear to be additive [28]. The question is raised whether cigarette smoking and use of oral contraceptives together have a synergistic effect on β-carotene plasma levels.

Association of Reduced Plasma Ascorbic Acid and β-Carotene Levels with Smoking, Human Papillomavirus Infection and Cervical Dysplasias

Plasma levels of the essential micronutrients ascorbic acid and β-carotene, associated with smoking and HPV infection, have been studied in 75 women referred to a colposcopy clinic for abnormal Pap smears. Each patient had a repeat Pap smear and a colposcopically directed biopsy. Cervicovaginal lavage and coded peripheral venous blood samples were obtained for HPV DNA hybridization studies and nutrient analyses, respectively.

Forty-five subjects had histopathologically diagnosed dysplasias of varying grades of severity. Among women with dysplasias, 53.3% were smokers. Sixty-six percent of subjects with and 34% without dysplasias were positive for HPV infection. The mean reduced plasma ascorbic acid, retinol, and β-carotene levels among the

dysplastic groups were comparable. A strong association between smoking history and reduced plasma ascorbic acid levels was noted independent of cervical dysplasias or HPV status. The findings underscore the importance of smoking, ascorbic acid and β-carotene as nutritional cofactors, and HPV infection in the pathogenesis of cervical dysplasias [29].

Antioxidant Nutrients in Lavaged Exfoliated Cervicovaginal Epithelial Cells

Saline lavage of the human uterine cervix yields *in vitro* samples of representative exfoliated cervicovaginal epithelial cells predominantly of the exocervix and vagina. Selected nutritional studies of such exfoliated cell samples have yielded several interesting findings: smoking and the presence of cervical intraepithelial neoplasia (CIN) increase the rate of exfoliation as determined by the numbers of cells counted in smokers compared to non-smokers in healthy women or between normal controls and those with CIN [30,31]; the cervicovaginal ascorbic acid levels in smokers and women with CIN are significantly lower than in healthy non-smokers [31,32]; the ascorbic acid level of exfoliated cervicovaginal cells in normal contracts is significantly greater than the leukocyte ascorbic acid concentration [31]; the β-carotene levels in plasma and in exfoliated cervicovaginal cell samples are both significantly reduced in patients with dysplasias and cervix cancer compared to control subjects ($p < 0.001$) [33]; and in women receiving a β-carotene supplement as part of a clinical trial, increased concentrations are detectable above baseline levels in exfoliated cell samples [33].

DISCUSSION

Several noteworthy aspects of the work reported here give credibility to the importance of antioxidant vitamins in the etiology and potentially in preventing progression of the oncogenic process attributed to high-risk HPV DNA infections. The protocols were, in general, designed to prospectively study a spectrum of multifactorial influences in the same patient; all historic data and laboratory samples obtained were coded, blindly determined and evaluated on all patients. This spectrum consisted of HPV DNA molecular hybridization and PCR assays of cervicovaginal lavage samples for HPV DNA typing, an epidemiologic health care-cancer risk factor questionnaire, a Block computer-designed nutritional survey, and coded peripheral venous blood samples obtained for HPLC assay of antioxidant nutrients. We measured baseline and in some cases sequential plasma and lavage concentrations of retinol, β-carotene, ascorbic acid and α-tocopherol by HPLC in samples initially acquired when women obtained Pap smears. In various protocols, we determined differences in smokers versus non-smokers, users of steroid contraception, and among patients with cervix dysplasias and cancer.

The results of our nutritional studies, interpreted within the constraints implicit in the continuum cytomorphologic hypothesis of the natural history of cervix cancer, support a conclusion that dietary antioxidant vitamins have a causative and hence protective role in the etiology and biology of cervix cancer. The vast majority of data bearing on the possible cancer prevention role of nutrients in humans are derived from observational and case-control epidemiological studies designed to probe possible antitumor effects of dietary intake. In the past decade, numerous reports indicated a strong correlation between dietary intakes of specific nutrients and reduced cancer incidence in various organ sites [16,34–38]. While the evidence from dietary surveys and laboratory studies is far from conclusive, lower dietary vitamin A and β-carotene intake and deficiency of intracellular retinol-binding protein are significantly associated with higher grade severe dysplasia and *in situ* cervix carcinoma. How dietary vitamin A and C intake and consumption of fresh fruits and vegetables relates to other established risk factors, such as HPV infection, smoking, or sexual activity is unclear. However, these nutritional leads require further investigation since β-carotene, α-tocopherol and ascorbic acid have long been recognized as having potent immunoenhancing properties [39,40].

Smokers have a significantly lower dietary intake of β-carotene, vitamin A, and ascorbic acid than non-smokers. We hypothesize that the effect of smoking on dysplasia is mediated in part via deficient vitamin C levels. Tobacco smoke contains mutagens and can generate free radicals. More severe grades of dysplasia were more

common in older women, suggesting that nutrition, as one of many influences over an extended period of time, may precede morphological dysplastic manifestations [27,28].

Free radical-induced damage is etiologically implicated in many chronic diseases, including cancer [18]. Active oxygen species are implicated *in carcinogenesis* and may be etiologically involved in the promotional phase when cascading interactions of gene expression modulate cell differentiation and growth. The intriguing quenching properties of various antioxidant nutrients that trap free radicals and singlet oxygen species appear essential to inhibit the generation and propagation of free radicals, thereby protecting mitochondrial oxidative function and inhibiting/preventing free radical-mediated lipid peroxidation and DNA damage.

The mechanism(s) underlying antitumor protective effects of carotenoids and other micronutrients are thought to involve antioxidant activities [17]. Epidemiologic and experimental data support an association between increased dietary intake of yellow-green vegetables and fresh fruits that are rich in antioxidant vitamins and protection against cancer incidence in various sites, including lung, stomach, pancreas, liver, colon, breast, and prostate [34,36,37,41–43]. High intake of dietary β-carotene has been reported to confer protection against ovarian and endometrial carcinomas [37,41]. Cervical cancer risk has consistently been demonstrated to increase with lower dietary intake of carotenoids as well as other antioxidants [44]. We reported decreased plasma levels of β-carotene, α-tocopherol and ascorbic acid in cervical cancer patients [24–26]. Nutrient-derived antioxidants, endogenous antioxidants, and related enzymatic mechanisms are involved in cellular defense integrity against free radical accumulation [45]. β-Carotene and possibly other non-provitamin A carotenoids, as well as vitamins C and E, are known biologically active antioxidants by virtue of their ability to quench free radical and singlet oxygen. High antioxidant capacity of these vitamin molecules shields DNA from oxidative damage and potential genotypic mutation events coincident with cancer initiation [17].

Pharmacologic antioxidant intervention can be a valuable chemopreventive addition to clinical management. Nutritional epidemiologic studies have not successfully identified specific dietary nutrients as having a cause-and-effect relationship in human cancers. There is no basis for suggesting that human cancer is a manifestation of vitamin deficiency. Nevertheless, 35–40% of cancer deaths can be attributed to diet; the devastating nutritional effects of advanced malignant disease are clinically too evident [38].

Sensitive and valid objective biomarkers as definitive endpoints are critically needed in the design of any prospective chemopreventive clinical trial. Current concepts of the pathogenesis and prevention of both cervix dysplasias and cancer are limited by incomplete data concerning the multifactorial etiology, pathophysiology, and molecular biology of the natural history of the neoplasm. Interactive nutritional results assume importance as biomarkers in the continuing effort to understand the multifactorial pathogenesis of cervix cancer and why some but not all HPV DNA subtypes have an oncogenic potential. The underlying molecular mechanism of cervix cancer biology needs to be elucidated to establish the credibility of the natural history, treatment, and prevention of cervix cancer.

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