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Micronuclei: A Potential Intermediate Marker for Chemoprevention of Aerodigestive Tract Cancer

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Abstract Because they may be used as a quantifiable estimate of the extent of recent DNA injury, micronuclei, extrachromosomal fragments of DNA, are among the most studied potential intermediate markers of cancer chemoprevention. Serial measurements of micronuclei frequency may be easily performed on scrapings from the oral cavity or on bronchial brushings. Assessment of micronuclei frequency and its response to chemopreventive agents has been incorporated into studies of upper aerodigestive tract and lung cancer chemoprevention. These studies have helped define the characteristics of micronuclei and have suggested a role for this test in future chemoprevention studies. Micronuclei frequency has been shown to be increased in the oral and bronchial mucosa of individuals with known carcinogen exposure and is higher at the site of the greatest carcinogen exposure, such as the site where tobacco quids are held, than in grossly normal-appearing mucosa. Treatment with chemopreventive agents leads to a reduction in micronuclei frequency. In oral leukoplakia studies, this effect followed treatment with β-carotene, retinol, α-tocopherol, and 13-*cis*-retinoic acid. The multistep process of epithelial carcinogenesis results from DNA damage and specific genetic events. That micronuclei reflect ongoing DNA injury suggests the hypothesis that long term suppression of cellular genotoxicity, as reflected by a reduction in micronuclei frequency, ultimately leads to a reduction in cancer incidence.

Key words: chemoprevention, intermediate markers, micronuclei

Assessments of micronuclei frequencies have been used in epidemiologic studies as a measure of carcinogen exposure [1–4]. The qualities that have made the micronuclei assay attractive for epidemiologic studies also apply to chemoprevention trials (Table I). Micronuclei, extranuclear fragments formed as a result of clastogen exposure, provide a quantifiable estimate of recent DNA injury [5]. In the epithelium, micronuclei frequency increases following carcinogen exposure and then declines as the cells containing micronuclei are sloughed. In the oral mucosa, the increase in number of micronuclei has been estimated to appear approximately 5–7 days following carcinogen exposure; the effect of a single exposure is expected to decline within 25 days [6]. The rate will, of course, vary with the degree of proliferation in the tissue at risk. Serial micronuclei frequencies may be used to assess changes in carcinogen exposure over time. The brief duration of the period from exposure to changes in micronuclei frequency makes the test well-suited for short term chemoprevention studies, since carcinogen exposure may translate into local DNA damage [7]. In the upper aerodigestive tract the assay may be used

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TABLE I. Micronuclei in Chemoprevention Studies of Oral Premalignancy

1. Quantifiable estimate of recent DNA injury
2. Noninvasive, allows for serial measurements
3. Chemopreventive effects studied in the organ of interest—oral mucosa
4. Frequency increased in individuals with known carcinogen exposure (*e.g.*, betel nut chewers, tobacco users)
5. Micronuclei also increased in "normal-appearing mucosa" from carcinogen-exposed individuals
6. Within individuals, micronuclei frequencies are greatest at the site with the most intense exposure (*e.g.*, where quids are held)
7. Micronuclei frequency declines with chemoprevention treatment (β-carotene, retinol, α-tocopherol, 13-*cis*-retinoic acid)

both to establish an estimate of risk and to determine whether the chemopreventive agent has a biological effect.

Another aspect of the micronuclei assay which is useful for chemoprevention studies; exfoliated cells from either bronchial brushings or oral mucosal swabs may be used to assess the impact of the chemopreventive agent within the epithelium of the organ of interest. In a short term chemoprevention trial it is extremely useful to determine whether the agent is exerting a protective effect on DNA damage within this tissue [8].

The prevalence of micronuclei has been shown to be increased in individuals exposed to carcinogens. In studies of oral premalignancy, micronuclei frequencies were higher in individuals who chewed tobacco or betel-nut quids or used smokeless tobacco than in individuals without these exposures [9–11]. Similarly, in a study of bronchial mucosal micronuclei frequency, specimens obtained from individuals who were active smokers had higher frequencies than those from individuals who had never smoked [12].

In U.S. studies of individuals without known exposure to the carcinogens tobacco or alcohol, micronuclei frequencies appear close to 0/1000 oral mucosa cells counted, and are generally less than 1/1000 cells counted in specimens from the bronchial mucosa. Micronuclei counts appear to be increased among individuals with oral premalignancy, but micronuclei remain an infrequent finding, in the range of 2–4/1000 cells counted [13]. Micronuclei counts from bronchial mucosa brushings of smokers are generally less than 8/1000 cells counted and typically less than 4/1000 cells counted. These oral micronuclei frequencies are lower than those reported in some studies of oral premalignancy that focus on groups with especially intense local carcinogen exposure, such as betel-nut chewers. However, methodologic inconsistencies in the non-U.S. studies may have led to the scoring of other indices of DNA damage in addition to micronuclei. Despite the differences in micronuclei frequency in these different populations, the same trends have been observed in different studies, that is, elevation of micronuclei frequency in exposed individuals and a reduction associated with chemopreventive agents.

MICRONUCLEI IN ORAL PREMALIGNANCY CHEMOPREVENTION STUDIES

Chemoprevention studies in oral premalignancy have demonstrated that administration of chemopreventive agents is associated with a reduction in micronuclei frequency. Stich *et al.* [14] observed a reduction in oral mucosa micronuclei frequency in Filipino betel-nut and tobacco chewers treated for 3 months with vitamin A (100,000 I.U./week) and β-carotene (180 mg/week) given in divided doses twice weekly. A decline in micronuclei frequency was observed in 37 of 40 study participants (93%). In a subsequent study of the efficacy of β-carotene or vitamin A alone on micronuclei frequencies in

betel-nut chewers, treatment with β-carotene resulted in micronuclei suppression in 22 of 25 participants (88%), with an overall mean decrease from 3.4% to 1.2% ($p < 0.001$) [15]. Vitamin A alone also suppressed micronuclei frequencies in 24 of 26 participants (92%), with an overall mean decrease in micronuclei frequency from 4% to 1.7% ($p < 0.001$). A study of β-carotene (180 mg/week) in Inuits living in the Northwest Territory of Canada also demonstrated a reduction in micronuclei frequency following treatment [16]. Assessment of micronuclei was incorporated into studies of oral leukoplakia in betel quid chewers in Kerala, India [17]. Study participants received placebo, β-carotene (180 mg/week) or a combination of β-carotene (180 mg/week) and vitamin A (100,000 I.U./week). Micronuclei counts were assessed in samples from both lesions and adjacent, clinically normal-appearing mucosa. Micronuclei frequencies were suppressed in both of the active treatment arms.

In a chemoprevention trial performed in China among a population at risk for development of esophageal cancer, the combination of retinol (50,000 I.U.), riboflavin (200 mg), and zinc (50 mg) reduced the frequency of micronuclei in the esophageal mucosa [18]. There were 0.19% micronucleated cells in the vitamin-treated group and 0.31% in the placebo-treated group ($p = 0.04$). In the same trial, however, micronuclei frequency in buccal mucosa cells was not reduced. The reduction in micronuclei following chemoprevention occurred in the site with the greatest risk of cancer.

In another oral premalignancy trial, treatment with 13-*cis*-retinoic acid was associated with a reduction in oral mucosa micronuclei [19]. Participants in this study received an initial 3 months of induction therapy with 13-*cis*-retinoic acid (1.5 mg/kg/day), followed by 9 months of maintenance therapy with either low dose 13-*cis*-retinoic acid (0.5 mg/kg/day) or β-carotene (30 mg/day). The micronuclei data for this recently reported trial are in preparation.

A recently reported trial of α-tocopherol in oral leukoplakia demonstrated that administration of this agent was associated with both clinical and histologic responses [20]. In addition, cell samples were obtained from both the normal-appearing mucosa and the lesions both at baseline and at the completion of therapy. Micronuclei frequencies were higher at the leukoplakia sites than in the normal-appearing mucosa; they declined at both sites following administration of the α-tocopherol.

METHODS

As with any potential intermediate marker, issues such as reproducibility, reliability, and interobserver variability are critical to the application of micronuclei frequency in chemoprevention studies. Measurements of micronuclei counts have been successfully incorporated into chemoprevention trials. The assay has provided consistent findings among research groups that have reported using this technique. Because micronuclei frequency is low relative to the number of cells counted, it is critically important that laboratories undertaking these investigations use meticulous techniques.

In our center, the procedure is done by first obtaining scrapings of the buccal mucosa or brushings from the bronchus and immediately smearing these specimens onto microscope slides [13]. The slides are sprayed with fixative (Adams Spray Cyte) and air-dried. The slides are then hydrolyzed in 1N HCl at 56°C for 10 minutes. The reaction is stopped by immersing the slides in chilled distilled H_2O for 1 minute. The slides are then immersed in Feulgen stain in a dark refrigerator for 90 minutes, washed in running tap water for 10 minutes, and counterstained with Fast Green for 45 seconds. The cells are mounted using Permount. The following criteria are strictly observed in counting micronuclei: a micronucleus must be less than one-third the size of the intact nucleus; micronucleus staining must be homogeneous and similar to that of the intact nuclei; and no karyorrhectic or pyknotic nuclei are scored.

In our studies, micronuclei frequencies are reported as the number of micronuclei observed per 1,000 cells counted, with a minimum of 500 evaluable cells necessary for the data to be incorporated into the study. Each slide is scored separately by an observer blinded to treatment assignment or timing of the specimens. When there are two slides for an individual, micronuclei counts for the slides are averaged.

Accurate counts obviously depend on the ability of the observers to reliably identify mi-

cronuclei. Using previously described criteria, cells should only be counted when they contain both intact nuclei and cytoplasm. We emphasize that extrachromosomal cytoplasmic DNA fragments which are scored as micronuclei are between 2–4 microns in size and have the same texture and intensity as the nucleus. In addition, the fragments scored as micronuclei must be in the same focal plane as the nucleus. Using these definitions, reliable micronuclei counts have been obtained. In the recent study of α-tocopherol in oral leukoplakia, two observers independently reviewed 10 slides in a blinded fashion. The Pearson correlation coefficient for the comparison of their micronuclei counts was 0.89.

SUMMARY

Clearly, micronuclei do not represent all nuclear abnormalities; but using careful techniques it is possible to obtain reliable measurements of micronuclei frequency and, consequently, a quantifiable estimate of recent DNA damage. Our rigid adherence to scoring criteria explains the lower prevalence of micronuclei reported in our studies than in some previously published reports. Although micronuclei frequencies are generally low in buccal mucosa cells obtained from the U.S. population, it is clear that they are elevated in carcinogen-exposed populations, and that they may be reduced following successful chemoprevention. Cumulative DNA damage is a critical element of multistep epithelial carcinogenesis, and a reduction in micronuclei frequency is believed to reflect a decrease in DNA damage. This raises the hypothesis that long term suppression of DNA injury, reflected in low micronuclei counts, may lead to reduction in cancer incidence. The correlation between long term suppression of micronuclei frequency and the prevention of invasive cancer needs to be demonstrated in prospective randomized studies, however. It is quite likely that markers of different stages of carcinogenesis, used in panels, will ultimately be necessary to accurately predict the risk of cancer development and to determine the impact of chemopreventive agents. Nonetheless, as a marker of recent DNA injury, micronuclei appear to be quite useful in oral leukoplakia chemoprevention studies.

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