Chemoprevention with Theaflavins of Rat Esophageal Intraepithelial Neoplasia Quantitatively Monitored by Image Tile Analysis

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Abstract

The objective of the study was to compare three methods of monitoring the inhibition by dietary theaflavins of N-nitrosomethylbenzylamine (NMBA)-induced rat esophageal intraepithelial neoplasia: the mean tile grade, measured by computer-assisted quantitative image tile analysis; tumor multiplicity; and mean tumor size. A “tile” is defined as a small portion of a microscopic image at $40 \times 87 \times 292 \, \mu m$ in size. The computer divided the image of esophageal intraepithelial neoplasia into a grid of contiguous tiles and measured four tissue features within each tile based on cytonuclear and tissue architectural changes used by pathologists to diagnose intraepithelial neoplasia. The tile grade is defined as the weighted sum of the four feature measurements within a tile, the weights being determined by Fisher linear discriminant analysis. The mean tile grade of 300 tiles is used to grade rat esophageal intraepithelial neoplasia. NMBA was given s.c., 0.5 mg/kg, three times a week for 5 weeks. Theaflavins were given in the drinking water at 360 ppm (low dose) and 1200 ppm (high dose) throughout the experiment. In a given set of four groups of rats, one group received theaflavins alone, one NMBA alone, one NMBA plus low-dose theaflavins, and one NMBA plus high-dose theaflavins. One set of four groups, four rats/group, was sacrificed at the 15th week and another at the 20th week after starting NMBA; a final set with 15 rats/group was sacrificed at 25 weeks. At the 15th and 20th weeks, the mean tumor grade was the only variable that responded significantly ($P < 0.01$) to the low dose of dietary theaflavins. In fact, tumor multiplicity and mean tumor size sometimes showed enhancement at these doses. At the 25th week, when there were 15 instead of 4 rats/group, the mean tile grade, tumor multiplicity, and mean tumor size were all significantly ($P < 0.01$) decreased by both low and high doses of theaflavins. The mean tile grade is a more sensitive and reproducible variable than tumor multiplicity and mean tumor size in detecting the chemopreventive effects of theaflavins on intraepithelial neoplasia in the rat esophagus. This suggests that the mean tile grade may be a useful intermediate end point for use in human chemoprevention trials.

Introduction

Tea (especially green tea) and its components have been reported in many publications to inhibit experimentally induced tumorigenesis in a variety of animal organ systems, including skin, mammary gland, lung, liver, forestomach, small intestine, and esophagus (1). In particular, studies have shown that green tea inhibits NMBA$^1$-induced rat esophageal tumorigenesis (1). Whole extracts of black tea are also inhibitory in this model system when given either during or after NMBA administration (1–3).

In the manufacture of green tea, the enzyme polyphenol oxidase is denatured by steam heat to prevent its activation by mechanical crushing of the tea leaves and subsequent catalytic action on the polymerization of catechins and gallic acid into oligomers, which are black in color. By contrast, in the manufacture of black tea, polyphenol oxidase is allowed to continue catalyzing the polymerization reaction until most of the catechins and gallic acid have been converted into black oligomers. Four of the oligomers, the agallate, two different monogallates, and the digallate, together termed “theaflavins” account for 3–6% of the total dry weight of black tea leaves (4).

In a previous report (5), the MTG, measured by CAQITA as described in detail below, was validated as a method for grading NMBA-induced rat esophageal carcinogenesis. The validation was performed in two ways: (a) the MTG was shown to correlate with the two universally used measures of response to a carcinogen, tumor incidence (the percentage of rats with esophageal papillomas), and tumor multiplicity (the number of esophageal papillomas per rat); and (b) the powerful chemopreventive agent, phenethylisothiocyanate, was shown to produce a reduction in MTG that closely paralleled the reduction it produced in esophageal tumor incidence and multiplicity (5).

As illustrated in Fig. 1, when rats were given NMBA s.c. at a dose of 0.5 mg/kg three times a week for 5 weeks, by the 10th week after starting the NMBA their esophageal epithelia exhibited diffuse intraepithelial neoplasia (6) plus multiple sharply demarcated microscopic plaques of high-grade intra-
Fig. 1. Effects of NMBA administration on rat esophageal epithelium. 
A, normal epithelium at 10 weeks. B, epithelium exhibiting mild dysplasia (diffuse intraepithelial neoplasia) at 10 weeks after the beginning of NMBA administration. C, epithelium exhibiting severe dysplasia (high-grade intraepithelial neoplasia) at 10 weeks after the beginning of NMBA administration. ×1000, stained with H&E.
epithelial neoplasia, each measuring up to 2 mm in length (5). At least some of these microscopic plaques in each rat progressed over the next 5 weeks into visible papillomas with high-grade intraepithelial neoplasia. The esophageal papillomas continued to increase in number and size until the 30th week, after which they blocked the passage of food through the esophagus, leading to the death of the rats. Wargovich et al. (7) reported that if the s.c. dose of NMBA is increased 7-fold to 3.5 mg/kg three times a week for 5 weeks, as early as the 15th week after starting NMBA, the papillomas in 17% of 29 rats had progressed to invasive squamous cell carcinomas. Thus, the microscopic plaques of intraepithelial neoplasia observed at the 10th week represent the beginning of a neoplastic continuum that ends in invasive carcinoma.

The objective of this study was to compare three variables for assessing the inhibitory effects of theaflavins on NMBA-induced rat esophageal carcinogenesis: the MTG, measured by CAQITA, tumor multiplicity, and mean tumor size.

**Materials and Methods**

**Chemicals.** NMBA was purchased from Ash Stevens (Detroit, MI). DMSO was purchased from Aldrich Chemical Co. (Milwaukee, WI). NMBA was analyzed for purity by reverse-phase high-performance liquid chromatography and proved to be >98% pure. A black tea polyphenol fraction containing 38.5% catechins, 13% theaflavins, and 1.3% caffeine was provided by the Division of Cancer Prevention, National Cancer Institute (Ogden Bioservices, Rockville, MD). The theaflavins in a 10% w/w boiling aqueous extract of black tea polyphenol fraction solids were precipitated by chilling the extract to 0°C and collecting the fractions by centrifugation. The precipitate was dissolved in boiling water and again precipitated and centrifuged. The precipitate was then dissolved in 1% potassium bicarbonate and extracted three times with equal volumes of ethyl acetate. The ethyl acetate was evaporated, and the solids were dissolved in water and applied to a preparative high-performance liquid chromatography column. Theaflavins were eluted in a linear gradient of 0–30% acetonitrile in water over 1 h. The fraction of intense absorbance at 450 nm was collected, lyophilized, and assayed to show that >90% of the dry weight of the precipitate consisted of the following theaflavins: theaflavin-3-gallate, theaflavin-3'-gallate, and theaflavin-3,3'-digallate. The remainder of the dry weight was considered to be made up of higher polymers of these theaflavins.

**Animals.** Male F344 rats were obtained from Harlan Sprague Dawley (Indianapolis, IN) at 5–6 weeks of age, housed in groups of three in plastic cages with hardwood bedding (Beta Chips; Northeastern Products Corporation, Warrenburg, NY), and quarantined for 2 weeks before use in experiments. All rats were fed a modified AIN-76A diet consisting of 20% casein, 0.3% dl-methionine, 52% cornstarch, 5% corn oil, 5% Alphacel, 3.5% AIN mineral mixture, 1% AIN vitamin mixture, and 0.2% choline bitartrate. Water was given ad libitum. The rats were maintained under standard conditions (20 ± 2°C, 50 ± 10% relative humidity; 12/12-h light-dark cycle). Animal cages and water bottles were changed twice a week.

**Preparation and Staining of Histological Sections.** At the time of sacrifice, rats were euthanized with CO2 inhalation and subjected to complete gross necropsy examination. The esophagus of each rat was excised, opened longitudinally, affixed to white index cards mucosal side up, and fixed in 10% neutral buffered formalin. The fixed esophagi were cut into three equal segments, which were oriented in paraffin blocks stacked flat one above the other such that a single microtome section showed a cross section of the entire wall of the esophagus. One histological slide of each esophagus was prepared and stained with the DNA-Feulgen stain. No counterstain was used.

**CAQITA.** The Bliss CAQITA system (Bacus Laboratories, Inc.) was used. This included a Zeiss Axiophot 2 microscope with an attached x, y microscope stage. All calculations described below were automatically performed by the computer. On the video monitor, the computer divided the image of the rat esophageal epithelium into a row of contiguous small image “tiles” that were fused seamlessly together and were not visible. Each tile measured 87 μm along the long axis of the esophageal epithelium and 292 μm perpendicular to the epithelium across the basal cell layer, intermediate cell layer, and part of the keratin layer. By using a mouse to “point and click,” the image tile registered at any point along the esophageal epithelium could be made to appear at magnifications of ×5, ×10, ×20, or ×40. The computer automatically made quantitative measurements of selected features (see below) in each contiguous image tile of the epithelium at ×40.

**Tissue Features Used to Calculate the Tile Grade.** The tile grade was calculated from measurements of four selected tissue features within each tile. The feature, “Sum OD per Tile;,” in absorbance units, is proportional to the total DNA within a tile and becomes increased in tiles containing piled-up neoplastic nuclei. The “Fraction of Tile Area Covered by Nuclei,” in μm², measures the silhouette area of aggregates of overlapping nuclei within a tile. The “Configurable Run Length with Step Length of 14.38 micrometers,” in hole counts per tile, is measured as follows. At ×40, there are >20,000 pixels within a tile. In relation to each given pixel (the index pixel), the computer measures the absorbance of a second pixel 14.38 μm away in both the horizontal and vertical directions. If the absorbance of either second pixel is less than the index pixel by 0.05 absorbance units or more, a “hole” is counted for the pixel pair. Increased hole counts occur when there is crowding and overlapping of nuclei within a tile. To measure “Deep Valley Detector;” in triplet holes per tile, the computer reviews all pixel triplets in the horizontal, vertical, and two diagonal directions. Pixel triplets whose center pixels have an absorbance less than either end pixel by 0.05 absorbance units or more are counted as a triplet hole. These occur at the borders of chromatin clumps, which are sharply margined, i.e., have a steep absorbance drop off, a feature that is characteristic of neoplastic change.

**Z-Score Transformation of Tissue Feature Measurements.** The measured value of each tissue feature within each tile was transformed into a statistical Z-score according to the equation, 
\[ Z = (x - \mu)/\sigma, \]
where \( x \) is the raw tile feature measurement from a carcinogen-treated rat, \( \mu \) is the mean of the same tile feature measured in normal rats, and \( \sigma \) is the SD of the same tile feature measured in normal rats (5).

**Calculation of Tile Grade.** Each tile was given a tile grade equal to the weighted sum of the four tile feature Z-scores, each weight being a unique coefficient <1, which remained constant among all tiles measured in a given esophagus (but which differed among different esophagi). The weighting coefficient was determined by Fisher linear discriminant analysis (8, 9).

The mean of 300 tile grades, or MTG, measured in a tissue section of neoplastic esophageal epithelium, provided a single numerical grade analogous to a pathological grade assigned by a pathologist. When the MTG is measured in a section of normal esophagus, a value near zero results. For example, the grand mean and SD of 12 esophageal MTGs measured in 12 normal rats at the 15th week after starting the studies was
0.025 ± 0.723, and in 12 rats at the 20th week it was 0.050 ± 0.626. The MTGs of rats that received theaflavins alone, without carcinogen, were between (−) 0.5 and (+) 0.5. When neoplastic rather than normal esophageal epithelium was analyzed, the MTG shifted to higher values.

**Studies.** The rats were acclimated on AIN-76 diet for 2 weeks and then randomly assigned to the different study groups. A regimen of the carcinogen, NMBA, was administered consisting of 0.5 mg/kg s.c. three times a week for 5 weeks. Theaflavins were given in the drinking water at a low dose of 360 ppm and a high dose of 1200 ppm, starting 1 week before administration of NMBA. For each set of four groups of rats, one group received theaflavins alone, one NMBA alone, one NMBA plus low-dose theaflavins, and one NMBA plus high-dose theaflavins. One set of four groups, at 4 rats/group, was sacrificed at the 15th week, and another set at the 20th week after starting NMBA; a final set with 15 rats/group was sacrificed at the 25th week. Esophageal tumors <0.5 mm were counted and mapped, and their sizes were measured as the product of two perpendicular diameters, in mm² (10). On one histological slide from each rat, CAQITA of the esophagus was performed, and a MTG was calculated, as described above.

**Statistical Procedures.** Three response variables were compared: the MTG, in SD units; tumor multiplicity, in number of esophageal papillomas per rat-bearing rat; and mean tumor size, in mm². By 15 weeks, esophageal tumor incidence was 100% in all groups receiving carcinogen. Thus, tumor incidence could not be used to discriminate between groups in this study. t tests were performed on the difference between means of groups of rats treated with NMBA alone (X₁), compared with groups treated with low or high doses of theaflavins (X₂) in the diet. In a conventional t test for significance of the difference between two sample means, the value of t is given by \( t = (X₁ - X₂)(n₁/n₂)\) where \( S_0 = [(S₁)^2 + (S₂)^2]/2 \) (11). Because the sample sizes were equal, this reduces to \( t = (X₁ - X₂)(n₁/n₂)\) where \( S_0 = [(S₁)^2 + (S₂)^2]/2 \) (11). The variable VNDM was defined as: VNDM = (X₁ - X₂)/[(S₁)^2 + (S₂)^2]1/2. The VNDM was used to analyze the responses of MTG, tumor multiplicity, and mean tumor size to theaflavins in the drinking water and also to determine the power of a t test to detect an observed or specified difference in means of groups of rats treated with NMBA alone (X₀) versus groups treated with low or high doses of theaflavins (X₁), at a null curve a level of 0.05 (12).

**Results**

**Inhibition by Theaflavins of NMBA-induced Rat Esophageal Intraepithelial Neoplasia Monitored by MTG, Tumor Multiplicity, and Mean Tumor Size.** Fig. 2 shows the effect of dietary theaflavins on the three response variables, MTG, tumor multiplicity, and mean tumor size, used to monitor the development of rat esophageal intraepithelial neoplasia after NMBA treatment. The MTG, averaged over the 15th, 20th, and 25th weeks, demonstrated a dose-dependent effect of theaflavins, responding with a 33% decrease at the low dose and a 42% decrease at the high dose.

At the 25th week, when there were 15 rats/group, dietary theaflavins at both low and high doses produced a significant reduction in the MTG and mean tumor size at the \( P < 0.01 \) level. With regard to tumor multiplicity at the 25th week, whereas the high dose of theaflavins produced a significant \( P < 0.01 \) decrease, the low dose produced no decrease at all. This observation, that the low dose of theaflavins at the 25th week produced a decrease in MTG and mean tumor size but not in tumor multiplicity, has a simple explanation; the low dose had no effect on the number of papillomas per esophagus, but it did reduce their size. On the other hand, the high dose produced a decrease in both the number and size of the papillomas.

Fig. 2 and Table 1 show that at the 25th week, in \( t \) tests...
comparing the groups given NMBA alone versus groups given dietary theaflavins, the observed difference in MTG, tumor multiplicity (1200 ppm dose only), and mean tumor size, were all significant at the $P < 0.01$ level with 99% power to detect them. At the 15th and 20th weeks, however, only the MTG showed a significant difference at both the low and high doses of theaflavins. The effects of theaflavins on tumor multiplicity and mean tumor size at 15 and 20 weeks were only significant ($P < 0.01$) in the case of tumor multiplicity at the 20th week.

Fig. 2 shows that the MTG of rats receiving NMBA alone declines between the 20th and 25th weeks. The most likely reason for this is that two of the four tissue features used to calculate the MTG depend on the area density of nuclei within the image tiles being measured. Visual inspection of the esophagai at the 25th week shows that the extent of reactive epithelial hyperplasia and subepithelial chronic inflammatory infiltrate attributable to carcinogen exposure for the first 5 weeks have diminished. The reduced degree of hyperplasia correlates with diminished. The reduced degree of hyperplasia correlates with the reduced area density of nuclei and, therefore, a fall in the MTG.

Table 1 gives the values at the 15th, 20th, and 25th weeks of theaflavins. The effects of theaflavins, the MTG had a VNDM ratio that was high (1.86) than that of tumor multiplicity (0.97) or mean tumor size (0.32). The low VNDM of mean tumor size (0.32), high variability of individual tumor sizes within each group, compared with the more moderate pooled variance levels of MTG (0.92) and tumor multiplicity (0.64).

**Discussion**

The results shown in Fig. 2 and Table 1 may be summarized as follows: (a) at the 15th and 20th weeks after initiating NMBA treatment when there were just four animals/group, only the MTG responded significantly to theaflavins ($P < 0.01$), whereas the responses of tumor multiplicity and mean tumor size were with two exceptions statistically insignificant and sometimes even showed enhancement; (b) analysis of the VNDM of the MTG, tumor multiplicity, and mean tumor size provided insight into the mechanisms of their response to the effects of theaflavins. Because of its greater sensitivity to the effects of theaflavins, the MTG had a VNDM ratio that was higher (1.86) than that of tumor multiplicity (0.97) or mean tumor size (0.32). The low VNDM of mean tumor size (0.32), on the other hand, was attributable to its high noise level, *i.e.*, high variability of individual tumor sizes within groups of rats; and (c) in addition to being the most sensitive chemoprevention response variable compared with tumor multiplicity, the MTG is a continuous parametric variable, scaled in SD units expressed to three significant figures, with practically unlimited range, whereas tumor multiplicity is a low number continuous variable with limited range, and mean tumor size is too variable to be useful unless large numbers of animals are used.

The variable MTG appears to have excellent promise for use as an intermediate end point in animals and, possibly, human chemoprevention trials. Because of its increased sensitivity, the MTG should be able to detect a greater difference in tissue biopsies and cytological smears before and after treat-

### Table 1: Inhibition by theaflavins of NMBA-induced rat esophageal carcinogenesis

<table>
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<th>Parameter</th>
<th>Week</th>
<th>Dose</th>
<th>$\bar{x}_1 - \bar{x}_2$</th>
<th>$\sqrt{S^2_1 + S^2_2}$</th>
<th>VNDM</th>
<th>$t$ test</th>
<th>Power</th>
<th>Extra $n$ for 99% power</th>
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<td>0.64</td>
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<td>1.23</td>
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<td>0.97</td>
<td>1.27</td>
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<td>0.92</td>
<td>1.86</td>
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ment of the same individual with a chemopreventive agent, thereby permitting evaluation by paired sample t tests with up to a 50% reduction in cohort size without significant loss of study power (13).

References
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