Urinary Excretion of N¹-Acetylspermidine and Other Acetylated Free Polyamines in the 1,2-Dimethylhydrazine Model of Experimental Rat Colon Cancer

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ABSTRACT

1,2-Dimethylhydrazine (DMH) is a potent procarcinogen with selectivity for the colon. Recently, it has been demonstrated that levels of N¹-acetylspermidine were elevated 2–3-fold in colonic tumors induced by this agent compared to control tissues. To determine whether alterations in the urinary levels of this acetylated polyamine or other polyamines were useful biochemical markers for colon cancer in this experimental model, rats were given s.c. injections of DMH (20 mg/kg body weight/week) or diluent for 26 weeks. Six weeks after the last injection, control and DMH-treated animals were placed in separate metabolic cages and their urines were collected for 24 h. The urinary levels (expressed as nmol/mg creatinine) of putrescine, spermidine, spermine, N¹-acetylspermidine, and N⁸-acetylspermine were then analyzed by high-performance liquid chromatography. Animals from each group were then sacrificed and their colons were examined for tumors.

The results of these studies demonstrated that the urinary level of N¹-acetylspermidine was an excellent biochemical marker for colonic tumors induced by DMH. At 18.3 nmol/mg creatinine, N¹-acetylspermidine was 100% sensitive and specific for colon cancer. Moreover, urinary levels of N⁸-acetylspermine were better for this purpose than the N¹-acetylspermidine/N⁸-acetylspermine molar ratio, a marker previously suggested to be more specific for certain cancers than free polyamines.

INTRODUCTION

Colon cancer is a major cause of mortality and morbidity in the United States and is diagnosed at an advanced stage in approximately one-half of patients (1). In order to develop better methods for the early detection of colon cancers, a number of laboratories have utilized various chemical carcinogens to induce colon malignancies in experimental animals (2, 3). One such carcinogen, DMH, as well as its metabolite azoxymethane have been used extensively for these purposes (3) and provide an experimental model which closely mimics human colon cancers in many clinical, pathological, and biochemical respects (2–4).

Alterations in the intracellular level of polyamines have been reported in human colon cancers (5–7) as well as in DMH- and azoxymethane-induced colonic tumors in rodents (8–10). Considerable evidence now exists that elevations in colonic intracellular polyamines secondary to increases in the biosynthetic enzyme, ornithine decarboxylase, may be intimately involved in the malignant transformation process of the colon in humans (5–7) and in experimental animals (8, 9, 11). While less extensively studied, certain lines of evidence also suggest that the enzymes involved in intracellular polyamine catabolism, particularly spermidine/spermine N¹-acetyltransferase, may also be involved in colonic carcinogenesis in humans and animals (4, 12). In 1984, Takenoshita et al. (12) reported a selective elevation of N¹-acetylspermidine, the product of spermidine N¹-acetyltransferase, in human colorectal adenocarcinoma. More recently, utilizing the DMH model of experimental colonic adenocarcinoma, increases in the level of N¹-acetylspermidine in treated distal "uninvolved" colonic tissue and 2–3-fold elevations of this acetylated polyamine in tumors of both colonic segments compared to control tissue have been reported (4).

Acetylated polyamines are major urinary excretory products (13) and urinary excretion of acetylputrescine and N¹-acetylspermidine has been shown to be increased in patients with various cancers (14–16) as well as in experimental animals with large tumor burdens (17). Information concerning the relationships between urinary concentrations of free and acetylated polyamines in colon cancer in humans and in experimental colon cancer, however, is sparse. It was, therefore, of interest to study the urinary levels of putrescine, spermidine, spermine, and N¹- and N⁸-acetylspermidine in control rats and rats given DMH for 26 weeks. The results described below demonstrate that the N¹-acetylspermidine, expressed as nmol/mg creatinine in 24-h urine samples, is an excellent biochemical marker for the presence of colon cancer in this experimental model and serves as the basis for the present report.

MATERIALS AND METHODS

Animals. Male albino Sherman rats weighing 100 g were given weekly s.c. injections of diluent or 1,2-dimethylhydrazine dihydrochloride (Sigma Chemical Co., St. Louis, MO) at a dose of 20 mg/kg body weight for 26 weeks as described (18). The animals were maintained on a pelleted diet (Camm Maintenance Rodent Diet; Camm Research Institute, Inc., Wayne, NJ) with water and food ad libitum. One week after the last injection, six animals from each group were placed in separate metabolic cages and their urines were collected under 2.0 M HClO₄ for 24 h. The animals in each group were then killed rapidly by cervical dislocation, their colons were excised and rinsed with iced saline, and the tumors were counted and weighed individually.

Histological Studies. All macroscopic lesions as well as at least two 1-cm specimens taken from proximal and distal uninvolved colonic segments were immediately fixed in 4% paraformaldehyde. Fixed specimens were then embedded in paraffin for light microscopic examination and stained with hematoxylin and eosin (18).

Free and Acetylated Urinary Polyamine Levels. The 24-h urinary samples from control and treated rats were collected, centrifuged at 10,000 × g for 30 min to remove precipitated proteins, and then filtered through 0.22-μm membranes (Millex-GV; Millipore, Bedford MA). Aliquots of 25–100 μl of the samples were then analyzed in duplicate for putrescine, spermidine, spermine, N¹-acetylspermidine, and N⁸-acetylspermidine by reversed-phase, high-performance liquid chromatography according to the method of Seiler and Knodgen (19) as described previously (10).

The compounds were identified by their relative retention times and quantified by comparison of peak areas to that of known amounts of standard. Peak area responses were linear over the range tested. No internal standard was used as recovery has previously been shown to be...
Quantitative (20). The identity of N'-acetylsperrmidine was further confirmed by acid hydrolysis and thin-layer chromatography as described previously by Matsuzaki et al. (21). Creatinine was measured in each sample as described previously (22).

Statistical Methods. Means of polyamine levels were compared between DMH-treated and control rats using a Student t test for independent samples (23). Sensitivity (probability of an elevated level in a DMH-treated rat) and specificity (probability of not having an elevated level in a control rat) were calculated using many different cutoff points. A plot of sensitivity versus 1 minus specificity (receiver operating characteristic curve) was generated for each polyamine variable measured (24). Curves were compared at the cutoff point where the "information content" was the highest. Information content was calculated using formulas derived from principles of information theory (25) and range from 1.0 (perfect discrimination) to 0.0 (no discrimination from the test). Animal weights and polyamine levels are expressed as mean ± SD and P < 0.05 was considered statistically significant.

RESULTS

General Observations. Initially, animals from both groups weighed approximately 100 ± 8 g. The final weights of the animals at 26 weeks were: control, 455 ± 20 g, N = 6; and DMH-treated, 441 ± 18 g, N = 6.

At sacrifice, the 6 control rats were not found to have any tumors in their colons. All 6 DMH rats, however, were found to have colonic tumors (3 proximal and 11 distal colonic adenocarcinomas) at this time. The majority of these tumors were moderately well differentiated and no tumor exceeded 1 g in wet weight. Two DMH-treated rats were also found to have distal colonic tubulovillous adenomas.

Urinary Polyamine Levels. Due to the variation in daily water consumption and urinary volumes, polyamine concentrations were widely scattered (data not shown). Because the weights between the two groups were not significantly different, and the 24-h excretion of creatinine is proportional to muscle mass, urinary polyamine excretion was best expressed as nmol/mg creatinine. As shown in Table 1, the 24-h urinary values (expressed as nmol/mg creatinine) of N'-acetylspermidine, putrescine, spermidine, and N'-acetyl spermidine + N'-acetylspermidine as well as the N'-acetyl spermidine/ N'-acetylspermidine ratio were all found to be significantly greater in DMH-treated animals compared to their control counterparts. Values of N'-acetylspermidine and spermine in the urine of these animals, however, were not significantly elevated. Urinary N'-acetylspermidine and N'-acetyl spermidine + N'-acetylspermidine levels showed the most significant differences between control and treated animals.

To further assess the usefulness of free and acetylated urinary polyamines in the detection of colon cancer in this experimental model, sensitivity and specificity were calculated. Table 2 shows the sensitivity, specificity, and information content for each polyamine level at the cutoff point where the information content is the highest. Several conclusions can be made based on the analyses presented in Table 2. First, N'-acetylspermidine urinary levels again appear to be the best discriminatory marker for colon cancer with a cutoff point for an elevated level of 18.3 nmol/mg creatinine. As shown, the N'-acetylspermidine + N'-acetylspermidine level has a high information content as does N'/N'-acetylspermidine, putrescine, and spermidine. N'-Acetylspermidine levels have less information content and spermine levels have little information content and are not adequate markers of colonic malignancy.

DISCUSSION

In 1971, Russell (26) was the first to document elevated levels of total and free polyamines in the urine of patients harboring a variety of tumors. Since that time, despite intensive investigation in this area, the clinical utility of this approach has been rather disappointing (27). The use of total or free urinary polyamines as biochemical markers for colorectal cancer in patients and in experimental animals also has shown little promise (28, 29). Thus, Horn et al. (28) and Carachi and Beeley (29) reported similar urinary polyamine levels in control patients and patients with colorectal cancer. The latter investigators (29), also utilizing the DMH model, noted only marginal differences in total polyamines in the urine of four control and seven carcinogen-treated rats. On the basis of these observations, Carachi and Beeley (29) therefore concluded that these urinary measurements were unlikely to be of value in the detection of colonic tumors in this experimental model.

In contrast to these earlier studies, the results of our experiments demonstrate for the first time that measurement of urinary levels of acetylated polyamines, particularly N'-acetylspermidine, are reliable markers for the presence of colonic
tumors in the DMH experimental model of colonic adenocarcinomas. At 18.3 nmol/mg creatinine, N\textsuperscript{1}-acetyl spermidine was 100% sensitive and specific for colonic tumors and was a perfect discriminator of disease.

Moreover, urinary levels of N\textsuperscript{1}-acetyl spermidine were superior to the N\textsuperscript{1}-acetyl spermidine/N\textsuperscript{4}-acetyl spermidine molar ratio, a marker previously suggested to be more specific for certain cancers than free polyamines (14-16). In agreement with prior studies in another experimental model of cancer (17), there was a tendency for N\textsuperscript{4}-acetyl spermidine to be increased in the urines of DMH-treated rats. This would appear to explain why this ratio was a less reliable urinary marker for colon cancer than levels of N\textsuperscript{1}-acetyl spermidine alone.

The present results are in general agreement with previous studies by Seiler et al. (17) in which elevations in N\textsuperscript{1}-acyl spermidine and the N\textsuperscript{1}-acetyl spermidine/N\textsuperscript{4}-acetyl spermidine ratio were detected in the urine of hepatoma-bearing Buffalo rats with large tumor burdens. This same group of investigators (30), however, failed to detect elevations in these polyamine parameters in the urine of rats with 7,12-dimethylbenzanthracene-induced mammary tumors. On the basis of these observations, they suggested that: (a) large tumor burdens (approximately 10–15 g of tumor) were necessary to create significant increase in N\textsuperscript{1}-acetyl spermidine and the N\textsuperscript{1}/N\textsuperscript{4}-acetyl spermidine ratio; and (b) the disproportionate increase in N\textsuperscript{1}-acetyl spermidine excretion seen in animals with tumor burdens was due to less efficient supply of the tumors with oxygen, thereby decreasing the rate of oxidation of acetylated polyamines by polyamine oxidase (17, 31).

In this regard, while the exact mechanism(s) responsible for the elevation in urinary N\textsuperscript{1}-acetyl spermidine levels in DMH-treated rats are unclear at this time, it should be noted that the number of tumors seen in these animals was rather small (2.33 tumors/rat) and no individual rat's tumor burden exceeded 3.5 g. Regardless of the mechanism(s) involved, it would appear that the measurement of acetylated polyamines, particularly N\textsuperscript{1}-acetyl spermidine, in the 24-h urine of DMH-treated rats is an excellent method for the detection of colon tumors induced by this carcinogen even in animals with relatively small tumor burdens. Since the urinary pattern of free and acetylated polyamines differ in rodents and humans (17), it is not clear whether such measurements will be as useful as a screening test for cancer.

The basis of our findings in this experimental model, our laboratory plans to address this potentially important clinical issue in the near future.

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