Differences in Colonic Nuclear Proteins of Two Mouse Strains with
Different Susceptibilities to 1,2-Dimethylhydrazine-induced
Carcinogenesis

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ABSTRACT

Comparisons were made of the prominent nuclear nonhistone proteins in the colonic epithelia of two mouse strains which differ markedly in their susceptibilities to tumor induction by 1,2-dimethylhydrazine (DMH). After 20 injections of DMH at weekly intervals, colorectal tumors occurred in 93% of SWR/J mice, whereas none occurred in AKR/J mice. Nonhistone nuclear proteins were extracted from nuclei isolated from the colonic epithelium of DMH-treated and control animals of both strains and from DMH-induced tumors of the SWR/J mice. The protein samples were analyzed by one-dimensional and two-dimensional gel electrophoretic techniques, and new methods of computer-assisted microdensitometry were used for graphical representation and quantitation of proteins in two-dimensional gels. Both one-dimensional and two-dimensional gel electrophoretic analyses revealed the presence of a number of prominent protein peaks in the tumor nuclei of DMH-sensitive SWR/J mice which were not evident, or present at much lower concentrations, in normal SWR/J or AKR/J colonic nuclei or in nuclei of the DMH-treated but tumor-free AKR/J mice. Conversely, the colonic tumor nuclei lacked two major proteins of the same molecular weight (M.W. 15,000) but different isoelectric points (pl 5.5 and 6.0) which were present in the nonmalignant colonic epithelial cells of both mouse strains. The results confirm the view that alterations in nuclear protein complement distinguish DMH-induced adenocarcinomas of the colon and suggest that the presence or absence of particular nuclear protein classes may signal changes leading to malignant transformation.

INTRODUCTION

Previous studies have established that the nuclear protein complement in cells of the colonic epithelium of CFN (Wistar) rats is progressively altered during the course of tumor induction by DMH (4). There is a striking increase in the nuclear content of 2 protein classes with molecular weights of approximately 44,000 (TNP₁) and 62,000 (TNP₂) which follows early, selective increases in their rates of synthesis (4, 5). Despite the narrow banding profiles of TNP₁ and TNP₂ in unidirectional SDS-polyacrylamide gels, both classes are complex when analyzed by 2-dimensional gel electrophoretic techniques (3). TNP₁ is predominantly localized in the dividing cell population of the tumor (2). It includes acidic DNA-binding proteins which are selectively released during brief digestions of tumor nuclei with DNase I, suggesting an association with transcriptionally active or newly replicating DNA sequences in the tumor nuclei (2, 3). In contrast, TNP₂ is predominantly found in the nondividing nuclei of DMH-induced tumors (2), and its components do not appear to have appreciable DNA-binding properties as judged by DNA affinity chromatography or protein release during limited DNase I digestions (3).

Proteins of the TNP₁ type have also been identified in human colonic adenocarcinoma nuclei and in a derivative cell line, HT-29, but the characteristic M.W. 44,000 peak was not evident in electrophoretograms from normal human colonic epithelia (2) nor in polyps from familial polyposis-affected patients at times when no sign of cancer had yet appeared (3).

In an attempt to further correlate specific changes in colonic nuclear protein complement with the malignant state, we have extended the analysis of nuclear proteins to strains of mice which differ in their susceptibility to tumor induction by DMH (6, 8). The genetic bases of these differences in DMH susceptibility have been discussed elsewhere (6, 9, 10). Our main consideration is the opportunity provided by the availability of DMH-sensitive and DMH-resistant strains to test whether the tumor nuclei contain characteristic protein “markers” not seen in the colonic epithelial nuclei of the DMH-treated but tumor-free resistant animals. One- and 2-dimensional gel electrophoretic techniques have been used to separate the nonhistone proteins extracted from nuclei of the normal colonic mucosa and DMH-induced tumors of the sensitive (SWR/J) mice, and the resulting electrophoretic patterns were compared with those of nuclear proteins from the colonic epithelium of control and DMH-treated mice of the resistant (AKR/J) strain. Because of the small quantities of tissue available for nuclear isolations, emphasis has been placed on the predominant nuclear proteins of the tumors and control tissues, and new methods for the quantitation and visualization of protein distributions in 2-dimensional gels were also applied. The results not only confirm that adenocarcinoma nuclei contain proteins that are absent or present in much lower concentrations in normal colonic nuclei, but they also reveal that the tumor nuclei are lacking in 2 major proteins which are prominent components of the nonmalignant epithelial cells of both mouse strains.

MATERIALS AND METHODS

Tumor Induction by DMH

SWR/J and AKR/J mice (obtained from The Jackson Laboratory, Bar Harbor, Maine), approximately 8 weeks old, were
used for all experiments. Tumors were induced in the sensitive (SWR/J) strain by s.c. injections of 15 mg DMH dihydrochloride per kg body weight at weekly intervals for 20 weeks. Mice of the same strain receiving injections of equal volumes of 0.9% NaCl solution for 20 weeks served as controls. All animals were killed 30 weeks after receiving the first injection of DMH or 0.9% NaCl solution. Similar injection protocols were applied to mice of the resistant (AKR/J) strain.

Isolation of Nuclei

Nuclei were prepared from portions (wet weight, 100 ± 25 mg) of the colonic epithelia of control SWR/J and AKR/J mice, from DMH-induced colonic tumors of SWR/J mice, and from the colonic mucosa of DMH-treated but tumor-free AKR/J animals, as described previously (4, 5). In all cases, the cells were broken by homogenization in 0.32 M sucrose-50 mM Tris-HCl buffer (pH 7.2)–25 mM KCl-5 mM MgCl2-1 mM CaCl2, and the nuclei were purified by centrifugation through a 2.2 M sucrose density barrier. The recovery of nuclei, based on the DNA content of the preparation relative to that of the initial homogenate, was approximately 65%.

Extraction of Nuclear Proteins

All nuclear preparations were washed twice with 10 volumes of 0.14 M NaCl containing 0.1 mM phenylmethylsulfonyl fluoride to inhibit protease activity. Acid-soluble nuclear proteins were removed by 2 extractions with 5 volumes of 0.25 n HCl. The residual nonhistone proteins were extracted by a modification of the method of Levy et al. (12) in 0.1 M sodium phosphate buffer (pH 7.4) containing 6 M urea, 0.4 M guanidine-HCl, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1% 2-mercaptoethanol. The proteins were separated from the DNA by centrifugation at 100,000 × g for 20 hr. The yield of NHNP from the various preparations was approximately 170 µg.

Electrophoretic Analyses of Nuclear Proteins

Separation of Nonhistone Proteins by Unidirectional Electrophoresis. All NHNP fractions were dialyzed extensively against 10 mM sodium phosphate buffer (pH 8.4) containing 0.1% SDS and 1 mM 2-mercaptoethanol. The protein samples, each containing 40 ± 10 µg, were applied to gels in a 10% polyacrylamide–0.1% SDS slab gel and separated according to size (11).

Separation of NHNP by 2-Dimensional Gel Electrophoresis. All NHNP samples were first fractionated on isoelectric-focusing gels essentially according to the method of O’Farrell (14) but using a different mixture of Ampholines (LKB Instruments, Inc.). The focusing gels contained 9 M urea, 4.5% acrylamide, 0.25% bisacrylamide, 2% Nonidet P40, and 2% total mixed Ampholines. The Ampholine mixture consisted of 3 parts covering the range (pH 5 to 8), 1 part (pH 8 to 10), 1 part (pH 3 to 6), and 1 part (pH 3.5 to 10). Electrophoresis of this mixture formed a nearly linear pH gradient over the range (pH 4 to 8) as measured by a pH-recording microelectrode-scanning unit (Bio-Rad Laboratories). After isoelectric focusing of samples (40 ± 10 µg) in the pH gradient, the proteins were separated according to size by electrophoresis in a 9 to 14% polyacrylamide gradient slab gel containing 0.1% SDS. The isoelectric-focusing gel was placed transversely across the top of the slab gel and bonded to it with hot 1% agarose in 50 mM Tris-HCl buffer (pH 8.4)-0.375 M glycine-0.1% SDS-5% 2-mercaptoethanol containing 0.1 M bromphenol blue as a tracking dye. (To avoid losses of protein, the isoelectric-focusing gels were not preequilibrated with the sample buffer before SDS-gel electrophoresis.) Electrophoresis was carried out using 50 mM Tris-HCl (pH 8.4)-0.375 M glycine-0.1% SDS-0.1 M 2-mercaptoethanol in the top reservoir and the same buffer minus mercaptoethanol in the lower reservoir. Electrophoresis in the second dimension was carried out at 20 mA/gel until the tracking dye reached the bottom of the gel (approximately 5 hr). After electrophoresis, the Ampholines were extracted from the gels in 50% trichloroacetic acid for 10 hr to reduce background staining. All gels were then stained in 0.1% Coomassie brilliant blue in 7% acetic acid-35% methanol-H2O, and excess dye was removed in the same solvent mixture.

Scanning and Calibration of Protein Patterns following Electrophoretic Separations

One-dimensional SDS-polyacrylamide slab gels were dried on sheets of dialysis membrane, and each lane was scanned at 575 nm in a Gilford recording spectrophotometer equipped with a linear transport device. Estimates of the molecular weights of individual nuclear protein bands were based on the mobility versus molecular weight plots for proteins of known molecular weight using the following as standards: myoglobin (M.W. 18,500); ovalbumin (M.W. 45,000); catalase (M.W. 55,000); and bovine serum albumin (M.W. 68,000).

Protein distributions in 2-dimensional gels were analyzed by microdensitometry of the photographic image (Fig. 1) using a high-speed rotating drum scanner (Optronix P1000; Optronics International, Inc., Chelmsford, Mass.). The output information (absorbance and X, Y coordinates) of individual protein spots was digitized and recorded on 9-track tape by a Kennedy

Table 1

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Treatment</th>
<th>No. of mice with tumors/ no. of mice examined</th>
<th>%</th>
<th>No. of colorectal tumors/mouse (Av.)</th>
<th>No. of other tumors and type</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWR/J</td>
<td>DMH for 20 wk</td>
<td>14/15</td>
<td>93</td>
<td>9.5</td>
<td>3*</td>
</tr>
<tr>
<td>SWR/J</td>
<td>Controls</td>
<td>0/20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AKR/J</td>
<td>DMH for 20 wk</td>
<td>0/13</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AKR/J</td>
<td>Controls</td>
<td>0/20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* One anal tumor and 2 lung tumors occurred together with colorectal tumors.
* Leukemia.

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RESULTS AND DISCUSSION

Different Response of SWR/J and AKR/J Mice to DMH Treatment. Although DMH is one of the most potent and reliable carcinogens for the induction of colonic tumors in rodents (7), differences in susceptibility to DMH have been observed in different inbred strains of mice (6, 8—10) and rats (1). Extreme differences in susceptibility to tumor induction by repeated weekly injections of DMH are displayed by SWR/J and AKR/J mice (9). The incidence and types of tumors found in the animals used for these studies are summarized in Table 1. An average of 9.5 colorectal tumors/mouse was found in the SWR/J strain after 20 weeks of treatment (killing the animals 30 weeks after the first injection). No colorectal tumors were present in control (0.9% NaCl solution-injected) SWR/J mice, and no tumors were observed in either the control or DMH-treated AKR/J mice.

NHNP of Colonic Epithelial Cells and DMH-induced Tumors. Nuclei were isolated from DMH-induced tumors and control colonic epithelial cells of SWR/J mice, and the nonhistone proteins were extracted and analyzed first by unidirectional SDS-polyacrylamide gel electrophoresis (11). Nuclei from control and DMH-treated AKR/J mice were also prepared, and their proteins were analyzed in parallel. The electrophoretic patterns of the 4 nuclear protein preparations are compared in Chart 1. The colonic nuclear proteins of control SWR/J and AKR/J mice (Chart 1, lower 2 panels) show very similar molecular weight profiles with some quantitative differences in the relative amounts of proteins with molecular weights of approximately 55,000, 78,000, and 95,000. Neither electrophoretic profile of the control animals shows the very prominent peak at M.W. 44,000 seen in the DMH-induced tumors of the SWR/J mice (Chart 1, top). This peak is also unamplified in the electrophoretic profile of the control animals. The failure of this protein class to accumulate in the nuclei of the DMH-treated AKR/J mice shows the extent of its potential as a 'marker' for malignant transformation in the colon.

A M.W. 62,000 protein class (TNP2) had also been noted as a prominent nuclear component of DMH-induced colonic tumors of the rat (4, 5) and in human adenocarcinomas (2). The presence of a similar peak in the colonic epithelial nuclei of DMH-treated but tumor-free AKR/J mice contraindicates its utility as a diagnostic marker for the presence of a colorectal tumor (although it may still be indicative of nuclear changes in the premalignant state).

Although the tumors of the SWR/J mice show the greatest dissimilarity in nuclear protein complement from the control epithelia, the nuclear protein composition of the colonic mucosa of the DMH-injected AKR/J mice is clearly altered by repeated injections of the carcinogen. While some of the changes observed might be indicative of a premalignant state, the altered electrophoretic profile of nuclear proteins in the...
DMH-treated AKR/J mice may also reflect changes in the cell population and cell kinetics of the colonic mucosa with corresponding alterations in the proportions of individual protein bands. It has been established previously that different cell types at different levels of the crypts in the colonic epithelium of the rat differ significantly in their NHNP composition (2, 5). Thus, alterations in cell kinetics in the DMH-treated AKR/J mice would be expected to change the electrophoretic profiles of the mixed nuclear proteins.

Other changes in the NHNP of colonic tumors became apparent with the more definitive protein separations achieved by high-resolution 2-dimensional gel electrophoresis. The proteins were first fractionated by isoelectric focusing in a pH gradient, and the components of the resulting bands were separated according to size by electrophoresis in polyacrylamide slab gels containing SDS (14). The 2-dimensional gel separations, in addition to their greater resolving power, provide 2 key parameters for each nuclear protein spot: its pl and its molecular weight. Because of the limited amounts of nuclear protein available (approximately 40 μg of total protein applied to each gel), emphasis has been placed on differences in the predominant NHNP of the colonic tumors and the nonmalignant mucosal cells of SWR/J and AKR/J mice. The staining patterns of the nuclear proteins of DMH-induced tumors and control colonic epithelial cells of SWR/J mice are compared in Fig. 1, upper panels. The corresponding displays of colonic nuclear proteins from DMH-treated and control AKR/J mice are shown in the lower panels of the figure.

These patterns were analyzed by raster-scanning microdensitometry using a computer program to provide a digital readout of the absorbance and position of each spot in the gel and to estimate protein concentrations by integration based on a 2-dimensional Gaussian least-squares fit (13). Fig. 2 shows one type of analysis of the protein distributions in each of the 4 vignetted areas depicted in Fig. 1. The spot intensities are plotted as a plane contour map in which protein concentration at a given locus in the gel is proportional to height as indicated by the concentric contour rings at those molecular weight and pl coordinates. The visualization and comparison of individual protein spots is further facilitated by a program which displays lines of data representing successive absorbance scans at 200-μm steps across the gel as shown in Fig. 3.

Comparisons of the protein distributions in the stained gels (Fig. 1) or their derivative computer displays (Figs. 2 and 3) reveal major differences between the nuclear protein complements of the SWR/J tumors and nonmalignant colonic epithelial cells. The tumor nuclei contain at least 6 components which are not evident in the nuclei prepared from the control tissues of SWR/J or AKR/J mice. These proteins, listed in order of increasing molecular weight using the convention M.W. × 10⁻³/pl, are 34.5/5.8, 44/7.0, 44/7.2, 46/6.25, 53/6.5, and 62/6.2. They are not detectable under these conditions in 2-dimensional gels of the nuclear proteins from DMH-treated but tumor-free AKR/J mice.

Both the tumors and the nonmalignant tissues share many nuclear proteins in common. These similarities will not be

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**Fig. 1.** Two-dimensional gel electrophoretic analyses of the colonic nuclear proteins of control and DMH-treated SWR/J (DMH-sensitive) and AKR/J (DMH-resistant) mouse strains. The NHNP were first separated by isoelectric focusing in cylindrical gels containing an Ampholine pH gradient. These gels were then bonded to slab gels containing SDS in a polyacrylamide gradient, and the proteins separated by electrophoresis at 90° to the original direction of migration. The positions of the stained protein spots are shown for: DMH-induced tumors in SWR/J mice (upper left); normal colonic epithelium of SWR/J mice (upper right); colonic epithelium of DMH-treated AKR/J mice (lower left); and normal colonic epithelium of AKR/J mice (lower right). The staining patterns were analyzed as shown in Figs. 2 and 3.
Fig. 2. Distribution of NHNP in 2-dimensional gels as visualized by computer-assisted raster-scanning microdensitometry of the staining patterns shown in Fig. 1. Vignetted areas of the gel, containing proteins in the size range of 14,000 to 70,000 daltons and pI's between pH 5.0 and pH 7.4, were selected for analysis. The computer program integrates densities by a 2-dimensional Gaussian least-squares fit and corrects for background. The spot intensities are plotted here as a plane contour map showing concentrations at the indicated molecular weight and pI coordinates. The patterns shown are for: DMH-induced tumors in SWR/J mice (upper left); normal colonic epithelium of SWR/J mice (upper right); colonic epithelium of DMH-treated AKR/J mice (lower left); and normal colonic epithelium of AKR/J mice (lower right).
Fig. 3. Computer analysis of the distribution of NHNP in 2-dimensional gels. Vignetted regions of the gels shown in Fig. 1, containing proteins varying in size from 14,000 to 70,000 daltons and with pI's between pH 5.0 and pH 7.4, were analyzed by raster-scanning microdensitometry. The digitized output is expressed as lines of data representing successive absorbance scans at 200-μm steps across the gel in the molecular weight dimension. The patterns shown are for: DMH-induced tumors of SWR/J mice (upper left); normal colonic epithelium of SWR/J mice (upper right); colonic epithelium of DMH-treated AKR/J mice (lower left); and normal colonic epithelium of AKR/J mice (lower right). Differences in the protein composition of tumor nuclei and nonmalignant cells are discussed in the text.

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discussed except to point out that the protein peak at 36/6.4 is a convenient standard of reference in all 4 gel patterns.

The colonic tumor nuclei are deficient in some proteins. Among them are proteins 35/4.6 and 34.5/4.7 and 2 major components at 15/5.5 and 15/6.0 which are present in all the nonmalignant nuclei but absent from the nuclei of the tumor.

The results confirm and extend earlier observations on the abnormal nuclear protein profiles of DMH-induced colonic tumors in the Wistar rat. They draw renewed attention to proteins in the M.W. 44,000 class which are present in the SWR/J murine tumors and not in the colonic epithelium of SWR/J controls or DMH-treated AKR/J mice. The 2-dimensional gel patterns reveal other easily identified additions and deletions in the tumor nuclei. The loss of the 2 major protein peaks at M.W. 15,000 is particularly striking. The nature of these proteins and their possible deletion from the nuclei of human colonic adenocarcinomas are now under investigation.

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