Correlation of DNA Methylation by Methyl(acetoxymethyl)nitrosamine with Organ-specific Carcinogenicity in Rats

Paul Kleihues, Gerhard Doerjer, Larry K. Keefer, Jerry M. Rice, Peter P. Roller, and Ruth M. Hodgson

ABSTRACT

Male Sprague-Dawley (Charles River CD) rats received a single carcinogenic dose (12 mg/kg) of the α-acetoxy derivative of dimethylnitrosamine, N-[14C]methyl-N-acetoxymethylnitrosamine, and were allowed to survive for 12 hr. Following i.v. injection, highest concentrations of 7-methylguanine and O6-methylguanine were present in DNA of the lung, the principal target organ in the carcinogenesis by N-methyl-N-acetoxyacetoxymethylnitrosamine at this dosage by this route of application. Injection i.p. of a similar dose of N-[14C]methyl-N-acetoxymethylnitrosamine led to preferential DNA alkylation in organs bordering the abdominal cavity, with highest levels of methylated purines in ileum and colon, the principal sites of tumorigenesis for this route of administration. Esterases potentially responsible for the bioactivation of N-methyl-N-acetoxyacetoxymethylnitrosamine in vivo were found in all organs investigated, with the highest levels of activity being present in rat kidney and liver. Incubation of N-[14C]methyl-N-acetoxymethylnitrosamine with DNA and esterases from rat kidney in vitro resulted in a pattern of methylated purines similar to that produced by N-methyl-N-nitrosourea and related methylating carcinogens, indicating that these agents, including dimethylnitrosamine, exert their biological effects through a common alkylating intermediate. Pretreatment of rat liver extracts with the esterase inhibitor diisopropyl fluorophosphate (10−4 M) reduced both the decomposition of N-methyl-N-acetoxymethylnitrosamine and DNA alkylation in vitro by more than 90%.

INTRODUCTION

The biotransformation of carcinogenic dialkylnitrosamines is thought to be initiated by hydroxylation at the α-carbon atom, yielding hydroxyalkyl-alkylinitrosamines as the proximate carcinogens. However, these intermediates are highly unstable and rapidly decompose to release an alkyl carbonium ion which reacts with various cell constituents. DMN-OAc is a relatively stable derivative of the α-hydroxy intermediate of the heptahydic carcinogen DMN. Administration i.p. of DMN-OAc to rats led predominantly to the induction of intestinal tumors (13, 33). Application i.v., on the other hand, produced primarily lung tumors (4, 11). In the present study, the methylation of DNA by [14C]DMN-OAc was determined in the intact animal under conditions previously shown to lead to tumorigenesis. We found that the route of administration greatly influenced the formation of methylated DNA bases in various rat tissues and that the extent of alkylation largely corresponded to the location of tumors induced by comparable doses of DMN-OAc. The esterase-catalyzed reaction of [14C]DMN-OAc with DNA in vitro resulted in a pattern of methylated purines identical to that produced by N-methyl-N-nitrosourea, supporting the view that the bioactivation of DMN is initiated by α-carbon hydroxylation and that both DMN and N-methyl-N-nitrosourea lead to the formation of a common alkylating species as the ultimate carcinogen.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley-derived CD rats were obtained from Charles River Breeding Laboratories, Wilmington, Mass. A standard laboratory diet and water were available ad libitum.

Chemicals. [14C]DMN-OAc was synthesized by Dr. E. J. Reist (SRI International, Menlo Park, Calif.) under contract with the National Cancer Institute Carcinogenesis Research Program from [14C]methylamine hydrochloride at a specific activity of 49 mCi/mmol.

\[
\text{CH}_3\text{NH}_2\cdot\text{Cl}^- + \text{H}_2\text{CO} + \text{CH}_3\text{CO}_2\text{H} + \text{NaNO}_2 \rightarrow \text{CH}_3\text{NCH}_2\text{---OCOCH}_3 + \text{NO}^\cdot
\]

The product was distilled at a bath temperature of 80° (20 mm) and condensed in a dry ice-acetone trap. Gas chromatography showed the presence of significant DMN, which was removed by evaporating a hexane solution of the distillate at 0° (20 mm) for 10 min. The overall yield of DMN-OAc as measured by UV spectrophotometry was 11%. A detailed description of the synthesis is in preparation. For injection, [14C]DMN-OAc was dissolved in phosphate buffer, pH 5.5 (0.15 m Na+), and unlabeled DMN-OAc was added to give a concentration of 2.4 mg/ml and a specific activity of 5.8 mCi/mmol. The radiochemical purity of the injection solution was checked by high-pressure liquid chromatography on a 4 x 30-mm C18-Bondapak column (Waters Associates, Inc., Milford, Mass.) eluted with 0.05 m sodium phosphate buffer (pH 5.5; flow rate, 2 ml/min) and found to be greater than 94%. On the same system, the chemical purity was 95% as measured by UV absorption at 254 nm. No radioactive contaminants eluted at the position of DMN. The label in [14C]DMN-OAc was confined to the methyl carbon atom to the extent of approximately 99%, as determined...
RESULTS

The stability of DMN-OAc in aqueous solutions was determined by recording the absorbance at 350 nm. Under physiological conditions (pH 7.2; 37°C), the half-life was found to be 26 hr. Heterolytic decomposition was markedly enhanced by the addition of a supernatant fraction from various rat tissues (Chart 1). The highest activity was present in kidney and liver. Extracts from the remaining organs were considerably less effective (Table 1).

The methylation of DNA bases was also determined in vitro, using [14C]DMN-OAc, calf thymus DNA, and a supernatant fraction from rat kidney. Chromatographic analysis of the acid hydrolysate revealed that of the total radioactivity the following proportions were present in methylated purines: 7-methylguanine, 58.2%; O6-methylguanine, 6.6%; 3-methyladenine, 8.1%. The calculated O6-methylguanine:7-methylguanine ratio was 0.11 (Table 3). Preincubation of rat liver supernatant with DIFP greatly reduced both esterase activity and DNA alkylation in vitro, with approximately 95% inhibition at DIFP concentrations of 10^{-4} M (Chart 2).

Following a single i.v. injection (12 mg/kg) of [14C]DMN-OAc, the highest concentrations of 7-methylguanine were present in lung DNA, followed by those in kidney, brain, and thymus (Table 2). Values for ileum, liver, stomach, and spleen DNA were one-fifth to one-eighth those of lung. In contrast, i.p. injection of a similar dose of [14C]DMN-OAc led to highest levels of alkylation in intestinal DNA, followed by liver, stomach, and spleen values. 7-Methylguanine concentrations in DNA of ileum and colon were 3 to 4 times higher than those in lung (Chart 3).

Similarly, concentrations of O6-methylguanine were highest in lung, kidney, and brain DNA after i.v. administration, whereas after i.p. injection this modified base was most extensively
produced in colon and ileum. In brain and thymus DNA, O\textsuperscript{6}-methylguanine concentrations were particularly low following i.p. injection of \[^{14}C\]DMN-OAc. In these organs, i.v. administration caused, respectively, 36 and 24 times more O\textsuperscript{6}-alkylation of guanine (Table 2).

**DISCUSSION**

Monofunctional alkylating agents react with DNA bases at various positions. The resulting pattern of alkylation products depends primarily on the chemical reactivity of the respective compound and is a major determining factor for the type of lesion induced in biological systems. There is increasing evidence that the initiation of malignant transformation by this group of carcinogens is related to their capacity for electrophilic attack at oxygen atoms in DNA bases. In particular, the extent of O\textsuperscript{6}-alkylation of guanine has been found to correlate closely with potential carcinogenicity in vivo (16, 20, 27). When DMN-OAc was incubated in vitro with DNA and a supernatant fraction from rat kidney, a pattern of alkylated DNA bases similar to that observed with N-methyl-N-nitrosourea (18) was produced. With both carcinogens, the O\textsuperscript{6}-methylguanine:7-methylguanine ratio was 0.10 to 0.11. Similar values have also been reported for 1-methyl-3-phenyltriazene and N-methyl-N\textsuperscript{\textprime}-nitro-N-nitrosoaniline (Table 3), suggesting that these carcinogens, irrespective of their different mode of activation, exert their biological effects via a common alkylating intermediate, probably methylidyazonium ion. Since DMN-OAc is the \(\alpha\)-acetoxyl derivative of DMN, our findings further support the hypothesis (7, 8) that the bioactivation of DMN is initiated by enzymatic \(\alpha\)-carbon hydroxylation. Of the compounds listed in Table 3, those with an O\textsuperscript{6}-alkylguanine:7-alkylguanine ratio of less than 0.1 show a weak (methyl and ethyl methanesulfonate) or hardly detectable (dimethyl and diethyl sulfate) carcinogenic activity. Potent in vivo carcinogens all have in common a ratio of 0.1 and above. An exceptional position is that of isopropyl methanesulfonate, which despite a relatively high proportion of oxygen alkylation is a very weak mutagen and carcinogen, probably due to the extremely low overall extent of DNA alkylation (18).

DMN and DMN-OAc differ considerably in their carcinogenicity for rats with respect to the type and location of tumors induced. Microsomal oxygenases capable of \(\alpha\)-carbon hydroxylating DMN are most abundant in liver and kidney. Since the half-life of N-methyl-N-hydroxymethylaminoimidazole is estimated to be less than 1 min and is possibly much lower under physiological conditions (26), a systemic distribution of this intermediate is possible only to a very limited extent, and this explains the almost selective induction by DMN of hepatic and renal neoplasms. Spontaneous cleavage of the \(\alpha\)-acetoxyl group of DMN-OAc in aqueous solution (pH 7.2; 37°) occurs very slowly with a half-life of 26 hr. However, heterolytic decomposition of DMN-OAc is efficiently catalyzed by ester-

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#### Table 1

**Determination of esterase activity in various rat tissues using DMN-OAc as substrate**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Esterase activity (milliunits/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>228</td>
</tr>
<tr>
<td>Liver</td>
<td>171</td>
</tr>
<tr>
<td>Lung</td>
<td>53</td>
</tr>
<tr>
<td>Colon</td>
<td>45</td>
</tr>
<tr>
<td>Thymus</td>
<td>37</td>
</tr>
<tr>
<td>Ileum</td>
<td>37</td>
</tr>
<tr>
<td>Brain</td>
<td>36</td>
</tr>
<tr>
<td>Spleen</td>
<td>35</td>
</tr>
<tr>
<td>Whole Blood</td>
<td>2</td>
</tr>
</tbody>
</table>

#### Table 2

**Concentration of methylated purines in DNA of various rat tissues 12 hr after a single i.v. or i.p. injection (12 mg/kg) of \[^{14}C\]DMN-OAc**

<table>
<thead>
<tr>
<th>Organ</th>
<th>7-Methylguanine</th>
<th>O\textsuperscript{6}-Methylguanine</th>
<th>O\textsuperscript{6}-Methylguanine:7-methylguanine ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>912</td>
<td>192</td>
<td>4.75</td>
</tr>
<tr>
<td>Kidney</td>
<td>817</td>
<td>108</td>
<td>7.56</td>
</tr>
<tr>
<td>Brain</td>
<td>673</td>
<td>27</td>
<td>24.93</td>
</tr>
<tr>
<td>Thymus</td>
<td>484</td>
<td>82</td>
<td>5.90</td>
</tr>
<tr>
<td>Colon</td>
<td>341</td>
<td>702</td>
<td>0.49</td>
</tr>
<tr>
<td>Ileum</td>
<td>183</td>
<td>633</td>
<td>0.29</td>
</tr>
<tr>
<td>Liver</td>
<td>155</td>
<td>450</td>
<td>0.34</td>
</tr>
<tr>
<td>Stomach</td>
<td>138</td>
<td>301</td>
<td>0.46</td>
</tr>
<tr>
<td>Spleen</td>
<td>119</td>
<td>224</td>
<td>0.53</td>
</tr>
</tbody>
</table>

#### Chart 2

Hydrolysis of DMN-OAc (\(\bullet\)) and in vitro alkylation of calf thymus DNA (\(\Diamond\)) by rat liver extract pretreated with various concentrations of the esterase inhibitor DIFP. The 100% value for the esterase-catalyzed decomposition of DMN-OAc was 192 milliunits per mg protein. As a measure of the extent of alkylation, 7-methylguanine was determined, with the control value being 0.64 mol % of guanine.
ases (26). These enzymes show a ubiquitous distribution in human and animal tissues (32). Using DMN-OAc as substrate and a postmitochondrial supernatant fraction as enzyme source, we found esterase activity in kidney and liver to be several times higher than that in other organs (Table 1; Chart 2). These authors postulate that, in addition to soluble esterases, there is a microsome-associated activity which, again, correlates with the results of carcinogenicity studies in rats (4, 11). In DNA of lung, kidney, and brain, the extent of O6-alkylation of guanine was 9, 8, and 36 times higher after i.v. injection than after a similar i.p. dose (Table 2). In contrast, i.p. injection of [14C]DMN-OAc resulted in a preferential alkylation of organs bordering the abdominal cavity, with the highest levels of alkylation being found in the target organs for this route of application. Concentrations of methylated bases somewhat lower than those in lung were present in kidney and brain DNA which, again, correlates with the results of carcinogenicity studies in rats (4, 11). In DNA of lung, kidney, and brain, the extent of O6-alkylation of guanine was 9, 8, and 36 times higher after i.v. injection than after a similar i.p. dose (Table 2). In contrast, i.p. injection of [14C]DMN-OAc resulted in a preferential alkylation of organs bordering the abdominal cavity, with the highest levels of alkylation being found in the target organs for this route of application (colon and ileum), followed by liver, stomach, and spleen. In all these tissues, the extent of DNA methylation was 2 to 3 times higher than liver, stomach, and spleen. In all these tissues, the extent of DNA methylation was 2 to 3 times higher than after i.v. administration. In liver DNA, the amount of O6-methylguanine was 12 times higher after i.v. injection (Table 2). This can be explained by the observation (15, 24, 25) that the enzymatic removal of this promutagenic base is dose dependent, with small amounts being more rapidly lost from hepatic DNA during the first few hr following the administration of the carcinogen.

The present experiments strongly indicate that a substantial
proportion of DMN-OAc administered i.p. is directly absorbed through the peritoneum by organs bordering the abdominal cavity. The possibility that the preferential induction of intestinal tumors is due to uptake of the carcinogen by the hepatic circulation and subsequent excretion via the bile seems less likely, since in this case a substantially higher extent of alkylation would be expected in small intestines than in the large bowel. Furthermore, this mode of distribution would not explain the higher concentration of methylated DNA bases in spleen and stomach following i.p. administration of [$^3$H]DMN-OAc.

In conclusion, both the carcinogenicity data and the in vivo DNA alkylation by [$^3$H]DMN-OAc indicate that the activity of esterases in various rat tissues is sufficiently high to activate a substantial proportion of the carcinogen at the site of application, in neighboring tissues, and in those organs which are first perfused following uptake of DMN-OAc into the systemic circulation. As with other carcinogens (6, 9, 22, 30), the present experiments also show that in species and tissues susceptible to the carcinogenic effect of alkylating carcinogens, the initial extent of alkylation is a significant factor for the incidence and location of tumors produced. In addition, the persistence of O6-alkylguanine and related promutagenic bases in DNA of target tissues may contribute to the organ-specific carcinogenicity of this group of chemical carcinogens (10, 14, 23, 27, 31). The possible role of the repair excision of O6-methylguanine from DNA in the carcinogenicity of DMN-OAc and the effect of agents modifying the activity of esterases in vivo are presently being investigated.

ACKNOWLEDGMENTS

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