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N-Nitrosocimetidine as an initiator of murine skin tumors with associated H-*ras* oncogene activation

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N-Nitrosocimetidine (NCM) is a derivative of the drug cimetidine, a methylguanidine derivative used in the treatment of peptic ulcer, and is known to be inactive as a complete mouse skin carcinogen, even when given in repeated high doses for a long period. In the current experiment, NCM was tested for its ability to initiate skin tumors on Sencar mice. It was applied at doses of 1 or 0.3 mg, 5 times/week for 6 weeks, followed by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 1 μg, 2 times/week for 50 weeks. Controls received acetone. The higher NCM dose had significant effects on TPA-promotable tumors, resulting in shortened time to first tumor, increased incidence of all tumors (2-fold) and of malignant tumors (4-fold), and greater tumor growth rate (2-fold), compared with the acetone/TPA-treated mice. The mice given the lower NCM dose did not exhibit increased tumor incidence, but their tumors had a significantly higher growth rate (3-fold) than those of the TPA controls. NCM without TPA treatment caused no tumors. Thus, NCM is a definitive, though weak initiator of TPA-promotable tumors. Nine tumors from the NCM-treatment groups were analyzed for activated oncogenes by the NIH 3T3 cell transfection assay. Five were positive and four of these were found by selective oligonucleotide hybridization analysis to have an A to T transversion in the second position of codon 61 of the H-*ras* oncogene. One of two tumors from the acetone/TPA group also contained transforming DNA and demonstrated this mutation. None of the tumors had a G→A transition mutation at the second position of codon 12 of this oncogene. Tumor initiation by NCM may then be associated with the same oncogene mutation reported for mouse skin tumors initiated by other types of carcinogens, although occurrence of the mutated oncogene in TPA controls precludes a definitive conclusion.

Introduction

N-Nitrosocimetidine (NCM*) forms readily by interaction of the widely-prescribed pharmaceutical agent cimetidine with nitrite under acidic conditions (1). The nitroso function of NCM is structurally similar to the potent carcinogen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), alkylates DNA *in vivo* and *in vitro* (2,3) and is known to be genotoxic *in vitro* (4–8). However, studies involving administration of NCM in the drinking water to rats or mice (9–12) or topical application to the skin of mice (10,13) revealed no significant tumorigenic effects. Investigations of the activation and detoxication of NCM have shown that the compound is stable in the absence of strong nucleophiles, but is rapidly decomposed by interaction with hemoglobin in the blood (14,15). A hepatic intracellular denitrosating activity dependent on glutathione has also been described (16). Cellular effects, including DNA alkylation, are presumably dependent on activation by intracellular nucleophiles such as glutathione (2,14). Since neoplastic cells may have an elevated level of glutathione (17), we postulated in a recent study that NCM might enhance rate of progression of mouse skin tumors from papillomas to carcinomas and confirmed experimentally that NCM did have such an effect, though with lesser potency than MNNG (13). This result raised the question as to whether NCM might also be an initiator of mouse skin tumors that could be promoted by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). A number of low mol. wt alkylating carcinogens, including urethane, β-propiolactone, 4-nitroquinoline-*N*-oxide, and triethylenemelamine (18–21) have this action. The outcome of our experiment to test this idea, presented here, confirms that NCM can initiate mouse skin tumors and that these frequently contain an activated H-*ras* oncogene.

*Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; NCM, *N*-nitrosocimetidine; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

Materials and methods

Animal treatment and tumor analysis

Female Cr:ORL Sencar mice, 8 weeks of age, were obtained from the Animal Production Area of the Frederick Cancer Research Facility and were housed under specific pathogen-free conditions, on hardwood shavings as bedding, 12/12 h fluorescent light/dark cycle, temperature 24 ± 2°C, humidity 50 ± 10%. They were given NIH 31 Open Formula Autoclavable diet and chlorinated or acidified tap water. They were shaved at least 2 days before topical skin treatment. NCM, prepared as described previously (12), was applied at a daily dose of 1 or 0.3 mg in 0.2 ml acetone, 5 times/week for 6 weeks. They were then treated 2 times/week with TPA, 1 μg in 0.2 ml acetone, for an additional 50 weeks. Controls were given NCM only, TPA only, or acetone. All groups consisted of 30 animals. Tumors were recorded and measured every 2 weeks. Nodules were recorded as papillomas when they achieved a diameter of at least 3 mm and persisted for at least 3 weeks. Animals were killed when morbid (showing clinical signs of disease or distress) or at 57 weeks after start of treatment. Representative sections of all lesions were classified histologically after hematoxylin and eosin staining. The remainder of all masses >5 mm were frozen at −80°C for oncogene analysis. Statistical analyses of the tumor incidence and multiplicity data and growth rates included the Student's *t* test (22), the Wilcoxon test (22), the Koziol test (23), the Kruskal–Wallis test (24), and the test for 2-tailed probabilities of Poisson variables (25). Two-tailed tests were used for incidence and multiplicity, and one-tailed tests for growth rates.

Oncogene analysis

High mol. wt DNA was isolated from tumors and control tissues and assayed for transfection of NIH 3T3 cells as described previously (26). For selective oligonucleotide hybridization, *Pvu*II digested DNA (10 μg) from the transfectants was fractionated in 1% agarose gels, which were then denatured, neutralized and dried under vacuum at 60°C. They were prehybridized with a solution of 5 × SSPE, 10 × Denhardt's solution, 500 μg/ml denatured salmon sperm DNA, and 0.1% SDS at T_d −5°C for 2 h. The oligonucleotide probes were antisense 19-mers encompassing codons 12 or 61 of c-H-*ras* and were ^{32}P-end-labeled using T4 polynucleotide kinase. They were hybridized overnight at T_d −5°C [T_d = 4° (G + C) + 2° (A + T)]. The probes were, for codon 12, wild-type (GGA): 5'-CCCACGCCTCCAGCGCCCA-3', mutant (GAA): 5'-CCCACGCCTTCAGCGCCCA-3'; for codon 61, wild-type (CAA): 5'-TACTCTTC-