Histogenesis and the role of p53 and K-ras mutations in hepatocarcinogenesis by glyceryl trinitrate (nitroglycerin) in male F344 rats

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Glyceryl trinitrate (GTN) was previously reported to induce hepatocellular carcinoma (HCC) in rats after prolonged feeding. The present experiments were undertaken to evaluate the histogenesis and molecular biology of these tumors and the possible role of nitric oxide (NO), a GTN metabolite, in their development. Male F344 rats received a single i.g. intubation of GTN (1.2 g/kg) at 6 weeks of age and/or a diet containing 1% GTN from 8 weeks of age until necropsy, i.e. for up to 78 weeks. Some animals were subjected to 2/3 partial hepatectomy (PH) at 9 weeks of age. Five sequential sacrifices (14, 32, 52, 78 and 84 weeks of age) were performed. No liver tumors developed in control rats or in rats that received GTN only by a single i.g. intubation, even when intubation was followed by PH. Preneoplastic foci, mainly of clear cell and mixed cell type (identified as positive for glutathione S-transferase placental form) were found from 14 weeks of age in rats receiving GTN in the diet. Focal eosinophilic areas (atypical foci) composed of atypical hepatocytes that often extended into the veins were observed beginning at 52 weeks of age. Some mixed hepatocellular adenomas and carcinomas arose in eosinophilic lesions. HCCs were seen beginning at 78 weeks of age, but only in rats receiving dietary GTN. Incidence of HCC in the latter animals was 50–75%. Most HCCs were well differentiated. The carcinogenic effect of GTN given in the diet was not affected by prior intubation of a large single dose followed by PH. No p53 mutations were found in 18 tumors but K-ras point mutations, all within codon 12, were found in 8/18 tumors, mostly those with cholangiocellular elements. These were first or second position G→T transitions or second position G→A transitions. While these mutation types have also been commonly seen in bacteria after NO-related DNA damage, the fact that tumors arose only on prolonged feeding of this potently bioactive agent at massive doses seems consistent with a more complex mechanism involving multiple (i.e. genetic and/or epigenetic) factors in carcinogenesis by GTN.

Introduction

Glyceryl trinitrate (GTN*) is a commercial explosive agent and widely used antianginal drug that has been shown to produce liver tumors on prolonged feeding to rats (1). Its pharmacological action has been attributed to its metabolic reduction to nitric oxide (NO) (2), a critical mediator of numerous normal and abnormal physiological phenomena (3–5).

Given the great industrial and medicinal importance of GTN, we wished to investigate the mechanism of its carcinogenic action, especially the possible role of NO. We had already noted that the distribution of point mutations induced in the DNA of Salmonella typhimurium strain TA1535 by GTN (6) was identical to that produced by a drug that spontaneously generates NO when dissolved in the culture medium (7), and reasoned that detection of a similar mutational spectrum in GTN-induced rat liver tumors might constitute evidence for the involvement of NO as a proximate genotoxic intermediate in the mechanism of carcinogenesis by GTN. Tissues from the previous feeding study (1) were no longer available, however, so we repeated the experiment in a modified form to obtain DNA for sequencing. We also included methodological variants of the original protocol that allowed us to examine the effects of regeneration versus normal homeostasis of liver tissue, as well as of long-term feeding versus single administration of GTN, on tumorigenicity.

Our results, described below, confirm the hepatocarcinogenicity of GTN on prolonged dietary administration to rats and reveal the presence of both G:C→A:T transitions and G:C→T:A transversions in the K-ras genes of more than a third of the resulting tumors. We also show that prolonged exposure to high dietary levels of GTN is required for production of tumors in this model, partial hepatectomy (PH) and high-dose single boluses having no detectable influence on tumor yield, and describe in detail the histogenesis of the tumors that did result from long-term exposure to this commercially important bioactive compound.

Materials and methods

Animals and chemicals

Male F344/Ncr rats were purchased from the NCI Division of Cancer Treatment's animal program at 5 weeks of age. Animals were housed at three to a polycarbonate cage with hardwood chips in an air-conditioned room with a 12 h light/12 h dark cycle and given diet (Meal no. 5010,Ralston Purina, Richmond, IN) and water ad libitum. Rats were maintained in accordance
Glutathione S-transferase placental form (GST-P), kindly provided by Drs.

Laboratories, Burlingame, CA) for immunohistochemistry. N. Ito and M. Sato, were used with the ABC Vectastain Elite Kit (Vector Laboratories, Inc., Burlingame, CA) for immunohistochemistry.

Animal experimentation

Two hundred male Fischer F344 rats were randomly divided into five groups, including 60 rats in group 1, 80 rats in group 2, 10 rats in group 3, 30 rats in group 4, and 20 rats in group 5. Rats from group 1 received a single i.g. intubation of 12 g SDM®-17 (1.2 g of GTN)/kg body wt at 6 weeks of age. Rats from group 2 received a single i.g. intubation of SDM®-17 at the same dosage as group 1 at 6 weeks of age, and were placed on test diet (1% GTN, 9% lactose) from 8 weeks of age for 76 weeks. Group 3 served as untreated controls and were fed powdered Purina Meal no. 5010 diet. The test diet was prepared once every 3 weeks and stored at 4°C.

DNA analysis for mutations

Thirteen of the tumors were frozen in liquid nitrogen at necropsy after preservation of a portion of each tumor for routine histology. Five additional paraffin-embedded tumors were also chosen for study after histopathological diagnosis. Tumor DNAs were examined for mutations in coding regions of the K-ras (exons 1 and 2) and p53 (exons 5 to 8) genes. After PCR amplification of the appropriate regions of DNA (using primers shown in Tables I and II) specimens were initially screened by single strand conformation polymorphism (SSCP) analysis for mutations. PCR from the original template and DNA from paraffin-embedded tumors, tumor tissue from one to four slides was extracted and determined as described previously (8), except that only a 4% alcohol wash with 100% ethanol was used following octane extraction.

Polymerase chain reaction

Exons 5, 6, and 8 of the rat p53 gene and exons 1 and 2 of the rat K-ras gene were individually amplified using the primers shown in Tables I and II. The p53 primers were intron-based, except for those for exon 67, which was amplified in two segments, each of which utilized one exonic and one intronic primer for PCR amplification. The upstream primer of K-ras exon 1 was intron-based, whilst the other K-ras primers were exonic. A 50-μl PCR reaction contained KCl (50 mM), Tris-HCl (10 mM, pH 8.3), dNTPs (1.2 mM), MgCl2 (1.5 mM), primers (0.2 μM each), template (1 μg for genomic DNA, 26 μl of extract for paraffin extract specimens), and Taq polymerase (1.25 U). A modified 'hot start' PCR was performed utilizing Taq-start antibody (Clontech, Palo Alto, CA) directed to the Taq polymerase, which rendered it inactive prior to heating the reaction to 72°C. To produce p53 amplification products, PCR reactions were heated to 94°C for 2 min prior to the following 30-cycle regimen: 92°C for 30 s, followed by annealing at 54°C for 30 s, 55°C (exon 6), 62°C (exon 7), or 61°C (exon 8) for 30 s, then extension at 72°C for 30 s. For exon 1 of K-ras, reactions received 30 to 40 cycles of PCR as follows: 92°C for 30 s, then 63°C for 30 s. For exon 2, the following three-cycle regimen was used for 30–60 cycles: 92°C for 30 s, 47°C for 30 s, 72°C for 30 s. Products were evaluated on 10% polyacrylamide–Tris-borate–EDTA (TBE) mini-gels (Novex, Inc., San Diego, CA).

Cold SSCP analysis

PCR products were washed three times with distilled water using Microcon 100 concentrators (Amicon, Beverly, MA) to remove primers and dNTPs and were resuspended in water in their original volume. The cold SSCP analysis was conducted according to Hongyo et al. (9) as summarized below. One to 5 μl of purified PCR product was added to a mixture of 0.4 μl of methylmercury hydroxide (Johnson Matthey Alpha Products, Ward Hill, MA), 2.0 μl of 15% w/v Ficoll loading buffer and 1X TBE sufficient to make a total volume of 20 μl. This mixture was heated to 85°C for 4 min then chilled on ice. The denatured mixture was subjected to electrophoresis in a 7 cm×10 cm×1 mm pre-cast 20% polyacrylamide TBE mini-gel. Gels were run at 300 V (43.5 V/cm, 26 mA) at a constant optimal temperature maintained by circulating buffer from a thermostatically-controlled circulator through both chambers of the gel cell. Optimal running buffer temperatures and times were as follows: 15°C for 6 h (p53, exon 5), 10°C for 6 h (p53, exon 67, 5′-segment), 19°C and 29°C for 1–2 h (p53, exon 67, 3′-segment), 18°C for 3 h (p53, exon 8), 20°C for 2.5 h (K-ras, exon 1) and 34°C for 4 h (K-ras, exon 2). Gels were stained with silver (Silver Express, Novex, Inc.) or with SYBR II (Molecular Probes, Inc., Eugene, OR).

DNA sequencing

Prior to sequencing, PCR products were gel purified by cutting the PCR fragment of interest from a 10% polyacrylamide TBE gel. The PCR fragment was eluted overnight in 100 μl of a solution 0.5 M in ammonium acetate and 1 mM in EDTA: DNA was precipitated with ethanol, then resuspended in 14 μl of distilled water. The purified PCR products were sequenced by dye-terminator sequencing, using a Sequenase Version 2.0 kit (United States Biochemical Corp., Cleveland, OH). Sequencing primers (Table I) were

| Table I. K-ras PCR and sequencing primers, oligonucleotide probes |
|-------------------|-------------------|
| Primer | Sequence |
| Exon 1 PCR | 5′-TAA GGC CTG CTG AAA ATG ACT GAG T-3′ |
| Exon 1 sequencing primer | 5′-TCC ACA AAA TGA TTA GAT A-3′ |
| Exon 2 PCR | 5′-AAG TAG TAA TTT AGG GAG AA-3′ |
| Exon 2 sequencing primer | 3′-AGA AAG CCC TCA CCA GTT CT-3′ |
| Oligonucleotide probe exon 12 (wild-type) | 5′-GGG GCT GGT GCC GTA GGC AA-3′ |
| Oligonucleotide probe exon 13 (wild-type) | 5′-ACA GCA GGT CAA GAG GAG TA-3′ |

| Table II. p53 PCR primers for rat |
|-------------------|-------------------|
| Target | Primer sequence | PCR fragment length |
| Exon 5 | 5′-GGTGACGTCTTCTGATTTTCGCT-3′ | 262 bp |
| Exon 67 | 5′-GGGACGTCTGACTTATCTTATTC-3′ | 158 bp |
| Exon 8 | 5′-GGGGGCTGTCTGACTTTCCTGCT-3′ | 198 bp |

with NIH guidelines. The animals were observed daily for abnormalities, and body weights were recorded once a week.

GTN was obtained from ICI Specialty Chemicals (Wilmington, DE) as a powder containing 10% nitroglycerin mixed with 90% lactose as diluent (product labeled SDM®-17) and stored at room temperature until it was mixed with intubation vehicle or feed. For intragastric (i.g.) intubation, 120 mg of GTN (1.2 g of SDM®-17) was suspended in 1 ml of corn oil. Test diet containing 1% GTN (10% SDM®-17) was prepared by mixing weighed quantities of SDM®-17 and powdered Purina Meal no. 5010 diet. The test diet was prepared once every 3 weeks and stored at 4°C.
Table III. Incidence of preneoplastic hepatocellular foci in male F344/NcCr rats treated with glyceryl trinitrate (GTN)

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aGiven a bolus of GTN by gastric intubation at a dose of 1.2 g/kg at 6 weeks of age.
bFed a diet containing 1% GTN from 8 weeks of age until necropsy.
cGiven a 2/3 partial hepatectomy at 9 weeks of age.
 dSignificantly different from group 1 at the P < 0.001 level.
 eSignificantly different from group 1 at the P < 0.01 level.
 fSignificantly different from group 1 at the P < 0.05 level.

Results

Clinical and necropsy findings

Untreated control rats (group 3) were consistently heavier and had lower liver weights relative to body weight than rats in any treatment group. Group 1 rats were only marginally (<10%) lighter than group 3 rats (e.g. 451 ± 16 g versus 483 ± 23 g at 78 weeks of age) and had comparable relative liver weights (~4% body wt). The mean body weights in groups 2 and 4 were consistently lower by 15–22% than those of group 1, and the mean body weight in group 5 was lower by 13–21% than that of group 1 from 54 weeks of age (experimental week 48) to the end of the experiment. No other clinical signs that could be related to GTN treatment were apparent in any of the rats.

Necropsies revealed hepatic tan-colored foci, reticular pattern and liver enlargement in rats from groups 2 and 4 beginning at 32 weeks of age. Tan and round or lobular nodules/masses were observed accompanying tan-colored foci in livers of rats from groups 2 and 4 at 78 and 84 weeks of age. Tan-colored foci were also found in livers of rats from group 5 at 84 weeks of age. The relative liver weights in groups 2 and 4 were higher than those of group 1 at each time point especially after 52 weeks (8.7–9.2% versus 4.0–4.4% in group 1).

Liver histopathology

Histopathological observations generally revealed hepatocellular degenerative lesions including glycogen accumulation in hepatocytes within centrilobular zones in rats from groups 2 and 4 (data not shown). This change was accompanied by the appearance of some apoptotic cells and became more severe and extensive with time. Incidences of preneoplastic hepatocellular foci are shown in Table III. Tiny preneoplastic foci including clear cell, eosinophilic or mixed cell lesions appeared early in groups 2 and 4 at 14 weeks of age (Figure 1). Mixed cell foci included a mixture of large clear and eosinophilic hepatocytes. The incidence of clear cell foci was consistently higher in groups 2 and 4 than in group 1 throughout the experiment. A similar trend was found for mixed cell foci. The incidences of eosinophilic foci in groups 2 and 4 were lower than those of clear cell and mixed cell foci. Basophilic foci were found in
Fig. 2. Atypical eosinophilic focus involving a central vein in the liver of a rat given a single i.g. intubation of glyceryl trinitrate (1.2 g/kg) at 6 weeks of age, fed a diet containing 1% GTN from 8 to 52 weeks of age, and partially hepatectomized at 9 weeks of age (group 2). GST-P immunohistochemistry ×100.

Fig. 1. Clear cell focus (A) H&E (×150) and GST-P-positive foci (B) immunohistochemistry (×150) in the liver of a rat given glyceryl trinitrate at 1% in the diet from 8 to 14 weeks of age (group 4).

Atypical foci were composed of either eosinophilic or basophilic hepatocytes. The incidences of each are presented in Table IV. The atypical eosinophilic focus was characterized by a population of hepatocytes that showed cytologic pleomorphism, prominent nucleoli, and an abnormal anatomical arrangement. This focus sometimes contained apoptotic cells and mitotic cells, was centrilobular or random in location within hepatic lobules, and was not expansive or nodular. The atypical basophilic focus was characterized by a population of hepatocytes that were slightly smaller than average and showed diffuse cytoplasmic basophilia and an abnormal anatomical

Fig. 3. Predominantly trabecular type of hepatocellular carcinoma with some gland formation in the liver of a rat given a single i.g. intubation of glyceryl trinitrate (1.2 g/kg) at 6 weeks of age, fed a diet containing 1% GTN from 8 to 78 weeks of age, and partially hepatectomized at 9 weeks of age (group 2). H&E ×75.

arrangement. Hepatocytes of both types of atypical foci often extended into the central hepatic vein (Figure 2). Atypical eosinophilic foci occurred in high incidence in groups 2 and 4 from 52 weeks of age. These were also found in 6/6 rats from group 5 at 84 weeks of age. Atypical basophilic foci were observed in groups 2 and 4 from 32 weeks of age, but no age-related increase in incidence was found.

Incidences of hepatocellular tumors are given in Table V.
Nitroglycerin hepatocarcinogenesis

Fig. 4. Hepatocholangiolar carcinoma in the liver of a rat given a single i.g. intubation of glyceryl trinitrate (1.2 g/kg) at 6 weeks of age, fed a diet containing 1% GTN from 8 to 84 weeks of age, and partially hepatectomized at 9 weeks of age (group 2). H&E X75.

Fig. 5. The margins of a hepatocellular carcinoma arising within an adenoma in a rat receiving a single i.g. intubation of glyceryl trinitrate (1.2 g/kg) at 6 weeks of age, fed a diet containing 1% GTN from 8 to 84 weeks of age, and partially hepatectomized at 9 weeks of age (group 2). H&E X75.

Tumors developed only in rats fed GTN in the diet (groups 2 and 4). Rats given only a single large i.g. dose (group 1) developed no tumors, even when dosing was followed by PH (group 5). The incidence of hepatocellular adenoma and carcinoma was age-related in groups 2 and 4. Hepatocellular adenomas were observed in groups 2 and 4 from 32 weeks of age and consisted of clear, eosinophilic, or mixed cell types. Hepatocellular carcinomas (HCCs), including carcinoma within an adenoma, were almost all well differentiated, and were either trabecular (Figure 3), glandular (Figure 4), solid, or variable mixtures of these morphologic types. HCCs arising within adenomas often contained areas of clear cells at the margins (Figure 5). HCCs developed in 50–75% incidences in groups 2 and 4 from 78 weeks of age. Incipient carcinoma was characterized by small focal lesions composed of hepatocytes that showed moderate atypia, slight basophilia, an abnormal anatomical arrangement and microglandular formations (Figure 6). These lesions appeared to arise from atypical eosinophilic foci. Incipient carcinomas were found in groups 2 and 4 from 78 weeks of age. Hepatocholangiocellular adenomas were observed in groups 2 (1/22) and 4 (2/4) at 84 weeks of age. Two hepatococholangiocellular carcinomas were found in a moribund rat from group 4 at 75 weeks and in another at 84 weeks. No mucin-secreting cells or argentaffin cells were found in these tumors.

Other non-neoplastic hepatocholangiocellular lesions were also found. Oval cell hyperplasia was observed in high incidence (45–100%) in groups 2 and 4 from 32 weeks of age. Age-related cholangiofibrosis was found (30–83%) in rats of group 4 from 32 weeks of age (Figure 7). In group 2, these lesions were observed in only one rat at 84 weeks of age.

**GST-P immunohistochemistry**

At 14 weeks of age, GST-P-positive hepatocellular foci appeared and were congruent with the preneoplastic foci identified by H&E staining in the livers of rats from groups 2 and 4 (Figure 1B). The positive foci were mainly located in the central zone. Foci that stained for GST-P were usually medium to large in size, but a single cell or a small cluster of hepatocytes was also occasionally found to be immunoreactive. The cytoplasm of normal hepatocytes surrounding GST-P-positive foci in GTN-treated rats was weakly positive. At 32 weeks of age, preneoplastic foci and hepatocytes in the central zone were GST-P-positive (Figure 8). These areas were congruent with the hepatocellular degenerative lesions. GST-P-positive foci were found both in the GST-P-positive central zone and randomly throughout the liver. GST-P-positive foci present in the central zone were difficult to distinguish from GST-P-positive populations of centrilobular hepatocytes. GST-P stained diffusely and intensely in the hepatocytes that comprised adenomas. At 52 weeks of age, a new population of GST-P-positive cells appeared that was more strongly immunoreactive than surrounding hepatocytes in the central zone (Figure 2). Those areas were identified as the atypical eosinophilic foci. At 78 and 84 weeks of age, hepatocytes in hepatocellular carcinoma or incipient carcinoma (Figure 6B) immunoreacted diffusely, intensely, or weakly for GST-P.

**Mutational analysis of K-ras and p53 genes**

Eight of 18 tumors (44%) examined had K-ras mutations, each of which was confirmed in duplicate PCR reactions. The total series of 18 tumors examined for mutations included eight carcinomas, nine adenomas and one atypical focus. The eight tumors with K-ras mutations included three carcinomas and five adenomas. All mutations were in codon 12 of exon 1. Four GTT→GTT transversions, three GGT→GAT transitions and one GGT→TGT transversion were found. All of these mutations could be detected by both ASOH and sequencing, except for two mutations (one GTT→GTT and one GGT→GAT), which were present in low relative abundance and were noted only as faint bands on ASOH, which was performed in duplicate on independent samples to confirm...
Mutations appeared to be more prevalent in specimens with histological evidence of glandular formation. Five of eight mutant samples had this histology, while the entire series of 18 had only seven samples with this histological pattern.

these results. The TGT and GAT mutations could also be detected by SSCP analysis. Figures 9 and 10 show representative SSCP and sequencing results. No codon 13 or exon 2 mutations were detected. No p53 mutations were detected.
other three mutant samples were either solid hepatocellular carcinomas (one case) or adenomas (two cases).

**Discussion**

We attribute all liver tumors that developed in rats fed diet containing GTN to the carcinogenic effect of GTN, which has been previously established (1). GTN induced liver tumors in male F344/NCr rats in this study when fed in the diet at a concentration of 1% together with 9% lactose. Ellis et al. (1) demonstrated the carcinogenicity of GTN for rat liver by feeding GTN specially formulated without lactose, but this preparation required the specialized services of an explosives plant. We elected to discount any modifying effect of lactose in test diet (at approximately twice its concentration in cow’s milk) on the carcinogenicity of GTN, and to ignore the fact that diets for the five groups were not isocaloric, since the purpose of the experiment was to study the tumors and their pathogenesis, not to quantify the tumorigenic effects. A bolus intragastric dose of 1.2 g GTN/kg body weight at 6 weeks of age was not carcinogenic even when followed by 2/3 partial hepatectomy at 9 weeks of age, and a bolus dose at 6 weeks of age followed by partial hepatectomy at 9 weeks of age did not affect the tumor yield that resulted from prolonged dietary administration of GTN. Our results confirm the hepatocarcinogenicity of GTN on prolonged oral administration to rats,
provide further information on the histogenesis of the tumors induced, and characterize mutations that occur in the K-ras oncogene, but not the p53 tumor suppressor gene, in these tumors.

Histologically, GTN induced progressive hepatic changes beginning with centrilobular hepatocellular degenerative changes followed by appearance of preneoplastic foci including clear cell, eosinophilic or mixed cell populations. These foci were, phenotypically, GST-P-positive and were demonstrated in rats as young as 14 weeks of age. Hepatocellular adenomas, detected in rats of group 4 after 32 weeks of age, consisted of clear cell, eosinophilic, and mixed cell types. Hepatocellular carcinomas with various morphologic types (i.e. trabecular, glandular or solid variants) arose within adenomas that contained areas of clear cells at the margins. Hepatocellular tumors thus appeared to originate from clear cell or mixed cell foci.

Atypical eosinophilic foci that often extended into adjacent veins were first seen at 52 weeks of age in rats receiving GTN in the diet and increased in incidence at later time points. These GST-P-positive atypical eosinophilic foci were regarded as a new type of cellular population, which arose within centrilobular areas. Such foci were not described by Ellis et al. (1) in their previous study (1), but the fact that they used Charles River CD rats rather than the F344/NCr rats used by us may account in part for this and/or other differences between results of the two studies. Atypical basophilic foci developed in rats of groups 2 and 4 from 32 weeks of age. These foci were not GST-P-immunoreactive. Such atypical basophilic foci appear not to progress to neoplasia. Additionally, in rats of group 4 at 78 weeks of age and in rats of groups 2 and 4 at 84 weeks there were focal lesions consisting of hepatocytes with moderate atypia, a slight basophilia, an abnormal anatomical arrangement, and microglandular formation that had histologic features suggestive of incipient carcinomas. Such focal lesions did not show nodular expansion, however.

Cholangiocellular lesions including bile duct proliferation and cholangiofibrosis were observed by Ellis et al. (1) in GTN-treated rats after feeding for 12 months. In the present study, oval cell hyperplasia and cholangiofibrosis were seen as early as 32 weeks of age in rats of group 4. The incidence of such lesions increased with age, particularly in group 4 rats, reaching 100% after 78 weeks of age. Furthermore, hepatocellular carcinomas and hepatocellular carcinomas were also observed in this group after 78 weeks of age. No cholangiocellular tumors were reported by Ellis et al. (1) in their study.

Point mutations in the K-ras gene were found in eight of the 18 tumors examined, all within codon 12 of exon 1. Of these, five were G→T transitions and three were G→A transitions. A similar distribution of mutations has been reported for the K- and N-ras genes of rat liver tumors induced by aflatoxin B1, but with relatively more G→A than G→T lesions (10/34 versus 4/34, respectively) (10-12). Ras mutations have been found less frequently in liver tumors induced by alkylating N-nitroso compounds; five of 93 hepatocellular carcinomas in rats initiated with N-nitrosomethyl(acetoxy)methylamine and promoted with phenobarbital exhibited codon 12 K-ras (all second position G→A) mutations, while none were seen in 54 liver tumors from rats given N-nitroso-N-methylurea or N-nitrosodiethylamine (12-14). There are to our knowledge no data on the presence or types of K-ras mutations in spontaneous rat liver tumors, probably because of their rarity.

While we can draw no conclusion as to whether our observed mutational spectrum resembles that of spontaneous rat liver tumors, we note a similarity to sequence changes seen on exposing DNA to the oxidation products of the GTN metabolite, NO. Roughly 2/3 of the mutations seen in bacteria transformed with plasmid exposed to peroxynitrite ion [ONOO⁻, formed in the reaction of NO with superoxide (15)] were G→T transversions (16), while G→A transitions predominated in similar experiments with agents that spontaneously released NO into the aerobic plasmid solution (17). Additionally, as mentioned above, G→A transitions were virtually the only mutations induced in Salmonella typhimurium when these bacteria were treated with either GTN or a spontaneous NO donor (6,7). Thus it is tempting to speculate that the K-ras mutations identified in the present study resulted from metabolic conversion of GTN to NO by reductive enzymes in the liver, and that the NO acted as a proximate tumor initiating agent that was further activated to DNA-damaging intermediates by both superoxide and oxygen.

Taken as a whole, however, our results seem more nearly consistent with a mechanism of action not solely reliant on genomic alteration as an initiating event. Single large doses of GTN failed to produce any liver tumors in this experiment, even when PH was subsequently performed to induce rapid proliferation of liver tissue. Indeed, prolonged feeding at high doses appears to be required for production of liver tumors by GTN in rats. While single small doses of GTN are efficiently converted to pharmacologically active metabolites on the first pass through the liver following oral administration (18), excessive exposure leads to the phenomenon of nitrate ‘tolerance’ [meaning that the more GTN is given, the less efficiently it is metabolized (19)], a condition involving numerous physiological effects that could influence tumor development. Because the reducing equivalents required for the conversion of GTN to NO [equation (1)] are furnished in part by glutathione and other compounds containing sulfhydryl groups (20), excessive GTN metabolism depletes the supply of these endogenous sulfhydryl compounds and diminishes their availability for xenobiotic detoxication and other critical metabolic functions (21). The hepatic enzyme family, the cytochromes P-450, can also supply reducing equivalents for GTN metabolism (22), and the NO thus produced can bind to the P-450 heme,
potentially inhibiting its drug-metabolizing activity (23). Chronic GTN administration also appears to stimulate endogenous vasoconstrictor mechanisms, leading to a physical dependence that on cessation of GTN dosage can produce rebound hemodynamic changes (24), including ischemia (25). Human volunteers given GTN experienced reduced sodium excretion, decreased hematocrit, and neurohormonal activation (26). There is evidence that tolerance to GTN is also associated with increased production of superoxide (27). Any NO produced metabolically from GTN in spite of our animals' presumably supertolerant state could react with this superoxide to form peroxynitrite (potentially accounting for the G→T transversions reported above), but it could also inhibit DNA repair (28,29) and induce apoptosis in various cell types (30–34). NO has also been shown to play a role in angiogenesis (35,36), hematopoiesis (37), and the adhesive interactions of neutrophils (38), platelets (39) and tumor cells (40). It seems likely that these two potential sources of metabolic dysregulation and other systemic perturbations could have a profound effect on tumor appearance and growth in animals fed massive doses of GTN for most of their lives. It should be noted that previous carcinogenicity studies in other species (dogs and mice) (1,41) as well as those involving chronic, low dose administration in rats (1,42) were negative, as was an earlier study of GTN as an initiating agent in mouse skin (43). The above considerations indicate that a role for DNA damage by nitric oxide cannot be inferred with confidence from our data, the similarity in mutational spectrum to those seen in bacterial expression systems notwithstanding. Instead, the results are consistent with the view that GTN may act through a complex mechanism involving multiple (genetic and/or epigenetic) factors.

\[ \text{RONO}_2 + 3e^- + 3H^+ \rightarrow \text{ROH} + \text{NO} + \text{H}_2\text{O} \]  

(1)

\[ \text{R} = \text{the 1,2- or 1,3-bis(nitrato)propyl group} \]

Epidemiologically, we have been able to locate little or no enduring evidence that GTN exposures present a carcinogenic risk to humans. There is a single case report of a patient developing a basal cell carcinoma on the portion of his skin where he had earlier been wearing a GTN patch (44), and one survey of patients taking GTN therapeutically showed a positive association (0.05 > P ≥ 0.01) with tumors of lung, trachea and bronchus (45). However, two earlier studies of the same large patient population showed no such correlation (46,47). Typical human doses range up to about 0.5 mg/kg per day of orally administered GTN (48), taken episodically, whilst our rats constantly ingested the compound at approximately 1000 times this rate beginning at 8 weeks of age. Occupational exposures in the explosives industry begin generally earlier in life and can be much larger than those sustained clinically, but we are aware of no epidemiological correlations with cancer in this arena, either (49,50). It would appear that the continuous, high-dose, lifetime feeding of GTN required to induce tumors in rats may be a special case not easily extrapolated to other species, especially to humans exposed clinically to orders-of-magnitude lower doses, and/or transiently to moderate levels in an occupational setting.

Acknowledgements

We thank Drs N.Ito and M.Sato for generously providing the GST-P antibody, and are grateful for the help of Kathy Breeze, Cindy Ferguson, Dan Logsdon, Craig Driver and Dee Green. The work was supported, in part, by USPHS contract NO1-CO-56000 to SAIC Frederick.

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