SHORT COMMUNICATION

Inhibition by neostigmine of hepatocarcinogenesis induced by N-nitrosomorpholine in Sprague-Dawley rats

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Several investigators (Kato & Shimazu, 1983; Lamar & Holloway, 1977) have suggested that liver regeneration after partial hepatectomy is affected by vagotomy. Maros (1970) reported that subdiaphragmatic vagotomy markedly suppressed cell proliferation after partial hepatectomy. These findings suggest that the parasympathetic nervous system is closely involved in hepatocarcinogenesis. Therefore, in the present work we examined in rats the effect of prolonged administration of the parasympathomimetic agent neostigmine methyl sulphate on hepatocarcinogenesis.

Fifty young (6-week-old) male Sprague-Dawley rats (SLC Inc., Shizuoka, Japan) were randomly divided into two groups and were treated as follows. Group 1 (25 rats) was given the vehicle only (plain olive oil) every other day until the end of the experimental week 18. From week 3, animals were also given drinking water containing 250 mg l⁻¹ of N-nitrosomorpholine (NNM; Sigma Chemical Co., St Louis, MO, USA) for 8 weeks. The NNM was dissolved in distilled water at a concentration of 50 g l⁻¹ and was stored in a cool place. The stock solution was diluted to 250 mg ml⁻¹ with tap water just before use, and 30 ml of this solution (which is less than any rat consumes in 48 h) were given to each rat from the bottles ad libitum. The bottles were replenished every other day after confirming that they were empty. When there was residual solution in the bottles, it was given intragastrically to control the dose of NNM. From week 11 until the end of the experiment, rats were given normal tap water only. Group 2 (25 rats) received alternate-day s.c. injections of neostigmine methyl sulphate (Sigma) at a dose of 0.1 mg kg⁻¹ body weight, as a suspension in olive oil. From week 3, animals were given NNM for 8 weeks in the same way as group 1.

At week 18, the rats (non-fasted) were killed by ether anaesthesia. The liver was immediately excised, and 2-mm slices obtained from the left lobe were immediately mounted on a brass chuck using OCT compound, frozen in dry ice-acetone (−86°C), and stored at −70°C. Serial 6 µm cryostat sections obtained from the frozen liver slices were also incubated in cold acetone (0–4°C) from 10 min and were stained with haematoxylin and eosin. Staining for γ-glutamyl transpeptidase (GGT) activity and adenylate cyclase activity was performed as described by Ruttenberg et al. (1969) and by Mayer et al. (1985), respectively.

Serial sections were scored for GGT-positive hepatic lesions and adenylate cyclase-positive hepatic lesions. The transactional area of the hepatic lesions in the plane of the tissue section and the area of the entire liver section were measured with a PIAAS LA-500 Personal Image Analyzer System (Tokyo, Japan). From the measured area of transaction of the lesions, the number of lesions per unit volume of liver was estimated by the method of Pugh et al. (1983), and the mean volume of the lesions per unit liver volume was calculated by the method of Campbell et al. (1982).

The labelling indices of the enzyme-altered hepatic lesions and the surrounding liver were examined in weeks 9 and 18. The labelling index was measured with an immunohistochemical analysis kit for assay of bromodeoxyuridine (BrdU) incorporation (Becton-Dickinson, Mountain View, CA, USA) (Gratzner, 1982; Morstyn et al., 1983) by a modification of the method described by Tada et al. (1985). To obtain the labelling index, the numbers of BrdU-labelled cells were counted among 500 cells in the surrounding liver and in enzyme-altered hepatic lesions of 0.7–1.2 mm largest diameter. The labelling index was expressed as the percentage of labelled cells per total number of cells examined.

Results were analysed by Student’s t test (Snedecor & Cochran, 1967). Data are given as means ± s.e. ‘Significant’ indicates a calculated P value of less than 0.05.

Five rats in group 1 and three rats in group 2 were killed in week 9 for examination of labelling indices of the enzyme-altered hepatic lesions and adjacent normal liver. No rats in group 1 died before week 18, but four rats in group 2 died before week 18. These were excluded from effective numbers.

Table I summarises the number, size and volume of GGT-positive and adenylate cyclase-positive hepatic lesions in NNM-treated rats. The two-dimensional data show that GGT-positive lesions and adenylate cyclase-positive lesions had a significantly smaller area (as per cent of parenchyma) in group 2 (neostigmine) than in group 1 (olive oil). Statistical analysis of the calculated volumetric data also shows that the number of GGT-positive hepatic lesions per cm³ and the volumes (as per cent of the parenchyma) of GGT-positive hepatic lesions and adenylate cyclase-positive hepatic lesions were significantly less in group 2 than in group 1.

Table II summarises the number, size and volume of hepatocellular carcinomas in NNM-treated rats. Both the observed transsectional data and the calculated volumetric data show that the incidence of hepatocellular carcinoma was significantly lower in group 2 (neostigmine) than in group 1 (olive oil).

Table III summarises the data on the labelling indices of pre-neoplastic hepatic lesions and adjacent normal liver for the NNM-treated rats. Group 2 rats treated with neostigmine had significantly lower labelling indices for pre-neoplastic hepatic lesions and adjacent liver than group 1 rats treated with olive oil only, at both times examined.

Acetylcholine has been demonstrated to be capable of influencing cell division (Byron, 1973). Gurkalo & Volfsen (1982) examined the effect of nicotine on the development of gastric cancers induced by N-methyl-N'-nitro-N-nitrosoguanidine, and found that parasympathomimetic agents inhibit carcinogenesis. However, thus far there have been no reports regarding the effect of cholinocceptor stimulation on hepatocyte proliferation.

Liver regeneration after partial hepatectomy is affected by vagotomy. Lamar & Holloway (1977) found that vagotomy at the cervical level significantly reduced the incorporation of labelled thymidine into liver DNA after partial hepatectomy. Maros (1970) found that liver cell proliferation after partial hepatectomy was markedly suppressed by subdiaphragmatic
vagotomy. Moreover, Kato & Shimizu (1983) reported that the increase in DNA synthesis after partial hepatectomy was suppressed by subdiaphragmatic vagotomy. Recently, however, Tanaka et al. (1987) examined the effects of hepatic vagotomy (sectioning of the hepatic branch of the vagus nerve) on liver regeneration after partial hepatectomy. These authors found that hepatic vagotomy delayed but did not suppress the increase in the rate of hepatic DNA synthesis. Kino (1988) also obtained similar results. In the present work, however, we found that prolonged alternate-day administration of neostigmine significantly reduced the labeling indices for pre-neoplastic hepatic lesions and for adjacent normal liver.

Our present results indicate that the parasympathetic nervous system may be closely involved in hepatocarcinogenesis although the dose of neostigmine was very high and the decrease in GGT positive foci relatively modest.

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