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Predictive Value of Rodent Forestomach and Gastric Neuroendocrine Tumours in Evaluating Carcinogenic Risks to Humans

Views and Expert Opinions of an IARC Working Group
Lyon, 29 November-1 December 1999

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IARC Working Group on Predictive Value of Rodent Forestomach and Gastric Neuroendocrine Tumours in Evaluating Carcinogenic Risks to Humans

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LIST OF PARTICIPANTS

Members

G.A. Boorman, Environmental Toxicology Program, National Institute of Environmental Health Sciences, PO Box 12233, Research Triangle Park, NC 27709, USA

C.C. Capen, Department of Veterinary Biosciences, College of Veterinary Medicine, Ohio State University, 1925 Coffey Road, Columbus, OH 43210-1093, USA

D. Chen, Department of Pharmacology, University of Lund, Sölvegatan 10, 22362 Lund, Sweden

E. Dybing, Department of Environmental Medicine, National Institute of Public Health, PO Box 4404, Torshov, 0403 Oslo, Norway (*Chairman*)

G.C. Hard[1], American Health Foundation, 1 Dana Road, Valhalla, NY 10595, USA

R. Herbert, Laboratory of Experimental Pathology, National Institute of Environmental Health Sciences, PO Box 12233, Mail Drop B3-08, Research Triangle Park, NC 27709, USA

M. Hirose, Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

M. Iatropoulos, New York Medical College, Department of Pathology, Basic Sciences Building, Valhalla, NY 10595, USA

M. Kidd[2], Gastrointestinal Clinic, E23, New Groote Schuur Hospital, Anzio Road, Observatory, 7925 Cape Town, South Africa

C. La Vecchia, Laboratory of Epidemiology, Istituto di Ricerche Farmacologiche 'Mario Negri', via Eritrea 62, 20157 Milano, Italy

I.M. Modlin, Yale University, School of Medicine, Department of Surgery/TMP 202, 333 Cedar Street, New Haven, CT 06520-8062, USA

G. Rindi[3], Anatomia Patologica 2, Universitá degli Studi di Brescia, Piazza Spedali Civili 1, 25124 Brescia, Italy

T. Sanner, Department of Environmental and Occupational Cancer, Institute for Cancer Research, The Norwegian Radium Hospital, 0310 Oslo, Norway

H.L. Waldum, Department of Intra Abdominal Diseases, Norwegian University of Science and Technology, 7006 Trondheim, Norway

G.M. Williams, New York Medical College, Department of Pathology, Basic Science Building – Room 413, Grasslands Road, Valhalla, NY 10595, USA

Representative/observer – Toxicology Forum[4]

Secretariat

R.A. Baan, Unit of Carcinogen Identification and Evaluation (*Co-editor*)

Y. Grosse, Unit of Carcinogen Identification and Evaluation

D. McGregor[5], Unit of Carcinogen Identification and Evaluation

C. Partensky, Unit of Carcinogen Identification and Evaluation (*Co-editor*)

J.M. Rice[6], Unit of Carcinogen Identification and Evaluation (*Head of Programme*)

J.D. Wilbourn[7], Unit of Carcinogen Identification and Evaluation (until 31 December 2000) (*Responsible Officer*)

Technical assistance

M. Lézère

S. Reynaud

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[1] Present address: 203 Paku Drive, PO Box 86, Tairua, 2853, New Zealand

[2] Present address: Yale University, School of Medicine, Department of Surgery/TMP 202, 333 Cedar Street, New Haven, CT 06520-8062, USA

[3] Present address: Dipartimento di Patologia e Medicina di Laboratorio, Sezione di Anatomia Patologica, Universita' degli Studi, via A. Gramsci, 14, 43100 Parma, Italy

[4] Unable to attend: P. Shubik, Toxicology Forum, 91A High Street, Oxford OX1 4BJ, United Kingdom

[5] Present address: 102, rue Duguesclin, 69006 Lyon, France

[6] Present address: 3213 Coquelin Terrace, Chevy Chase, MD 20815, USA

[7] Present address: 16, rue des Charmes, 69680 Chassieu, France

INTRODUCTION

The *IARC Monographs* Programme is an international consensus approach to the identification of chemicals and other agents and exposures that may present carcinogenic hazards to human beings. The *Monographs* assess the strength of the published scientific evidence for such identifications, which are based primarily on epidemiological studies of cancer in humans and on bioassays for carcinogenicity in mice and rats. Information that may be relevant to the mechanisms by which the putative carcinogen acts is also considered in making an overall evaluation of the strength of the total evidence for carcinogenicity to humans.

In the evaluations of carcinogenicity made by *IARC Monographs* working groups, results of bioassays for carcinogenicity in experimental animals are generally considered to predict a carcinogenic hazard for humans. The Preamble to the *Monographs* affirms that '**in the absence of adequate data on humans, it is biologically plausible and prudent to regard agents and mixtures for which there is *sufficient evidence* of carcinogenicity in experimental animals as if they presented a carcinogenic risk to humans.'** *Sufficient evidence* is considered to exist when a working group agrees that 'a causal relationship has been established between the agent or mixture and an increased incidence of malignant neoplasms or an appropriate combination of benign and malignant neoplasms (a) in two or more species of animals or (b) in two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. Exceptionally, a single study in one species might be considered to provide *sufficient evidence* of carcinogenicity when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset.' The Preamble adds that 'the possibility that a given agent may cause cancer through a species-specific mechanism which does not operate in humans should also be taken into consideration.'

Following a meeting on mechanisms of carcinogenesis in risk identification held in 1992 (Vainio *et al.*, 1992), the Preamble to the *IARC Monographs* was revised to formulate precisely how mechanistic evidence should be used in overall evaluations of carcinogenicity to humans (IARC, 1992). Since then, several IARC publications have dealt with specific topics on generic mechanisms of carcinogenesis that are relevant to overall evaluations of carcinogenic hazards of certain groups of chemicals to humans. These include reports on peroxisome proliferation and its role in carcinogenesis (IARC, 1995), on mechanisms of carcinogenesis by inhalation of fibres (Kane *et al.*, 1996), on the use of short- and medium-term tests for carcinogens and data on genetic effects in carcinogenic hazard evaluation (McGregor *et al.*, 1999), and on interspecies differences in thyroid, kidney and urinary bladder carcinogenesis (Capen *et al.*, 1999). Criteria for the application of specific kinds of mechanistic evidence have been formulated and are included in the Summary Reports of scientific experts which are included in these documents. These reports have subsequently assisted *IARC Monographs* working groups in evaluations and re-evaluations of certain substances (e.g., IARC, 1997, 1999).

In the *IARC Monographs* system of classification, an agent with *sufficient evidence* of carcinogenicity in experimental animals and *inadequate evidence* of carcinogenicity in humans will ordinarily be placed in *Group 2B — possibly carcinogenic to humans*. When there is strong evidence that carcinogenesis in experimental animals is mediated by mechanisms that do operate in humans, the agent may be upgraded to *Group 2A — probably carcinogenic to humans.* More than half of the agents and exposures currently (November 1999) in Group 2A are so classified on the basis of contributory mechanistic evidence that reinforces *sufficient evidence* for carcinogenicity in rodents, and two agents (ethylene oxide (IARC, 1994) and neutron irradiation (IARC, 2000)) have in this way been classified in *Group 1 — carcinogenic to humans*. However, the classification scheme also allows for down-grading into *Group 3 — the agent is not classifiable as to its carcinogenicity to humans* if there is strong, consistent evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans or is not predictive of carcinogenic risk to humans. In a relatively few well-documented cases (e.g.,_*d*-limonene, saccharin and its salts, melamine (IARC, 1999)) downgrading from Group 2B to Group 3 has occurred on the basis of such evidence.

Numerous chemicals can induce tumours of the forestomach epithelium in rats and/or mice and occasionally in other rodent species when given orally by admixture in the diet or as bolus doses by gavage, or when given by other routes of administration (see Dybing & Sanner in this volume). Many such substances have been evaluated in the *IARC Monographs* (see Appendix 1 in this volume). Some of these clearly act primarily through genotoxic (DNA-reactive) mechanisms, and often induce tumours of the forestomach together with glandular stomach neoplasms and/or neoplasms at non-gastric sites. For some other agents that induce tumours of the forestomach, however, evidence exists that the mode of carcinogenic action may include some non-genotoxic mechanism(s). The predictive value for assessment of human cancer hazard of tumours arising in such circumstances could be different from that of histologically similar neoplasms that arise in the forestomach following exposure to substances whose primary mode of action is through a genotoxic mechanism.

In addition to these forestomach tumours, gastric neuroendocrine tumours have been shown to be induced in rodents by an increasing number of substances. Unlike squamous-cell tumours of the forestomach, gastric neuroendocrine tumours have an exact counterpart in human beings. These tumours are rare in humans, however, and their causation is not well understood.

This IARC workshop was held in Lyon during 29 November–1 December 1999 to examine the mechanisms by which tumours of the forestomach and tumours of gastric neuroendocrine cell origin occur in rodents, and to consider the predictive value of these tumours for the identification of carcinogenic hazards to humans, when they occur alone and when they occur along with tumours in other organs. The workshop did not formally evaluate the carcinogenic hazard posed by any agent; such evaluations are undertaken by *IARC Monographs* working groups.

In the following sections, etiological risk factors in rodents for both the above-mentioned tumour types are summarized and evidence for the possible carcinogenic mechanisms involved is discussed. For rodent gastric neuroendocrine tumours, this was done in the context of what is known concerning the corresponding human tumours. Hypotheses underlying multi-species and species-specific mechanisms are summarized, the applicability of these mechanisms to humans is discussed, and current gaps in our knowledge of them are highlighted. Finally, for both tumour types, recommendations are presented on how the mechanistic data could be used in overall evaluations of carcinogenicity to humans.

Summary Reports at the beginning of each section use the background scientific reviews and case studies that were prepared before the workshop by individual participants. These individually authored papers formed the basis for the discussions that were held and are also included in this volume.

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SUMMARY REPORT

Squamous-cell tumours of the forestomach

In rodents, including the mouse, rat and Syrian hamster, the stomach is divided into two parts by the mucoepidermoid junction separating squamous from glandular epithelium. The proximal part, or forestomach, is non-glandular, forms a continuum with the oesophagus, and is lined by keratinized, stratified squamous epithelium. The distal part, or glandular stomach, empties into the duodenum and is lined by a specialized glandular epithelium. The forestomach is separated from the glandular stomach by a grossly visible, elevated fold, the limiting ridge. This contrasts with the anatomy of the human stomach, the lining of which is entirely glandular, the human mucoepidermoid junction occurring where the oesophagus joins the stomach. There is thus no forestomach in humans.

Tumour pathology

Benign and malignant squamous-cell tumours of the forestomach have been thoroughly described in mice (*Mus musculus*; Leininger & Jokinen, 1994), rats (*Rattus rattus*; Takahashi & Hasegawa, 1990) and Syrian hamsters (*Mesocricetus auratus*; Takahashi & Okamiya, 1996). The tumours are histologically similar in all three species.

Benign squamous-cell papillomas of the forestomach are exophytic growths that may vary greatly in size and may grow progressively to occupy almost the entire lumen of the forestomach. They may be single or multiple, sessile or papillomatous, warty or cauliflower-like in gross appearance. They do not invade the gastric wall and do not metastasize. Microscopically, they usually consist of branching outgrowths of well-differentiated keratinizing stratified squamous epithelium, with a fibroepithelial core and a well-arranged basal layer of cells. Small papillomas may be difficult to distinguish from focal regenerative epithelial hyperplasia.

Malignant squamous-cell carcinomas of the forestomach are characteristically invasive and may ulcerate. Microscopically the carcinomas are characterized by sheets, nests or cords of neoplastic squamous keratinized epithelial cells that invade the underlying muscularis and beyond. Well differentiated squamous-cell carcinomas may resemble papillomas histologically except for the invasion of the underlying structures, and distinction from papillomas may be difficult. Poorly differentiated carcinomas have cellular and nuclear atypia and are often non-keratinized. Distant metastasis, especially to regional lymph nodes, may occur.

Pathogenesis. The forestomach is a storage organ colonized by microflora and subjected to changes in pH. These factors may have some role in the pathogenesis of tumours induced by specific agents. Chemicals given orally come into direct contact with the forestomach epithelium, sometimes at high concentrations and for prolonged periods.

Proliferative or hyperplastic changes occur in the forestomach epithelium in response to the agents administered. Reactive changes commonly involve the limiting ridge that separates the squamous and glandular epithelia. Reactive epithelium may become papillomatous and difficult to distinguish microscopically from early benign tumours. Squamous-cell carcinomas may progress from pre-existing benign tumours or may evolve directly from altered mucosal epithelium.

Background incidence. Squamous-cell papillomas and carcinomas are extremely rare in untreated rats (Goodman *et al.*, 1979). Historical control data from the National Toxicology Program show that squamous-cell papillomas of the forestomach occur at a low incidence in feeding studies in Fischer 344 rats: males, 2/1354 (0.1%); females, 2/1351 (0.1%), while squamous-cell carcinomas have not been recorded: males, 0/1354; females, 0/1351. In $B6C3F_1$ mice squamous-cell papillomas of the forestomach are more common: males, 18/1355 (1.3%); females, 20/1353 (1.5%), while squamous-cell carcinomas are rare: males, 1/1355 (0.1%); females, 0/1353 (Haseman *et al.*, 1998) (see also Boorman *et al.*, Methyl bromide, this volume). In Syrian hamsters, the occurrence of benign and malignant tumours of the forestomach is much more frequent than in rats and mice, with reported incidences of squamous-cell papillomas of 4.1% in females and 6.1% in males, and of squamous-cell carcinomas in 0.5% of males (Dontenwill *et al.*, 1973).

Etiology: Benign and malignant tumours of the forestomach in mice and rats have been produced by about 12% of 144 chemicals given orally by gavage in bioassays reported by the National Toxicology Program, while only 2% of chemicals given by other routes of administration produced such tumours. In the majority of cases forestomach tumours occurred in conjunction with increased tumour incidences at non-gastric sites and occasionally with tumours of the glandular stomach. Of 120 chemicals that induced forestomach tumours, the forestomach was the only target site affected in 16% of the cases, usually after oral administration by gavage (see Dybing & Sanner, this volume).

Among forestomach carcinogens that are genotoxic, diethyl sulfate is an example of a chemical that produces only forestomach tumours when given to rats by gavage in corn oil while 1,3-butadiene is a multisite carcinogen that causes forestomach tumours in male and female mice but not in rats when given by inhalation (IARC, 1992; 1999). In the case of diethyl sulfate, the carcinogenicity may be restricted to the forestomach after oral dosing only because the compound is not distributed to other tissues in significant amounts. It induced local sarcomas but apparently no distant tumours in rats after subcutaneous injection (whereas the arachis oil vehicle did not). In addition, all in-vivo genotoxicity assays with diethyl sulfate have been conducted using intraperitoneal administration (see IARC, 1992, 1999; Pletsa *et al.*, 1999).

The following is a summary of the more important toxicological features of those chemicals that have been reviewed in detail in the first part of this volume. These chemicals were chosen to illustrate some of the patterns of forestomach tumour development that occur during carcinogenicity experiments in rodents.

Benzofuran (see Williams & Iatropoulos, this volume)

Human exposure to benzofuran can occur during coke production, coal gasification, the production of coumarone–indene resins, the combustion of coal and during exposure to tobacco smoke.

When administered orally in corn oil by gavage to male and female mice and rats, benzofuran induced a non-dose-related increase in the incidence of forestomach papillomas in $B6C3F_1$ mice, mainly in males but also in low-dose (120 mg/kg bw) females. Squamous-cell carcinomas were induced only in male mice of the low-dose group (60 mg/kg bw). No forestomach tumours were found in Fischer 344 rats. In mice, squamous-cell hyperplasia was increased in all dose groups except low-dose males. Since squamous-cell papillomas were increased in low-dose male mice as well as in the other mouse groups, there would appear to be no correlating preneoplastic lesion from which the papillomas might have been derived. In male and female mice there were dose-related increases in pulmonary and hepatocellular adenomas, which showed higher incidence in males than in females at the high dose, but not at the low dose. In male mice hepatoblastomas were induced, and in female rats renal tubular-cell adenocarcinomas.

Benzofuran was not mutagenic to *Salmonella typhimurium*. It did, however, induce mutations at the thymidine kinase locus of L5178Y mouse lymphoma cells in the absence of metabolic activation. It also induced sister chromatid exchange but not chromosomal aberrations in Chinese hamster ovary cells. Thus benzofuran is not clearly DNA-reactive *in vitro*, but has the potential to interfere with chromatid integrity. No in-vivo genotoxicity studies have been reported for this compound.

No data on the absorption, disposition, metabolism and excretion of benzofuran are available. It induced changes in activities of biotransformation enzymes in female CD-1 mice, with increases in epoxide hydrolase, glutathione_*S*-transferase, 7-ethoxycoumarin deethylase, NADH-quinone reductase and UDP-glucuronosyl transferase, and reduction in activity of cytochrome P450-associated enzymes.

In 13-week in-vivo studies with rodents, histological changes observed after treatment with benzofuran were a minimal hepatocellular necrosis and renal tubular necrosis in rats and renal tubular necrosis and regeneration in male mice, but no forestomach changes in either species.

The pathogenesis of none of the observed tumours has been satisfactorily explained. It is speculated that the underlying combined factors operating to produce forestomach papillomas in mice at very high, life-long exposure to a daily bolus of benzofuran might include a higher pH in the forestomach through stimulation of the parasympathetic nervous system, a longer transit time, more abundant microflora and various degradation products of benzofuran.

Butylated hydroxyanisole (see Williams_& Iatropoulos, this volume)

Butylated hydroxyanisole (BHA) has been used as an antioxidant in foods and cosmetics as well as in pharmaceuticals, rubber and petroleum products.

When given in the diet, BHA induced squamous-cell papillomas and carcinomas in the forestomach in male and female Fischer 344 (and male Sprague-Dawley) rats, male Syrian golden hamsters and male $B6C3F_1$ mice. In Japanese house musk shrews, which do not have a forestomach, no gastric proliferative lesions were found in a 72-week feeding study with high dietary concentrations of BHA.

Most routinely performed in-vitro mutagenicity assays have produced no evidence of mutagenic activity of BHA. The *Staphylococcus aureus* gene mutation assay without bio-activation and the chromosomal aberration assay in Chinese hamster cells with metabolic activation gave positive results with this compound. The available in-vivo DNA interaction assays were negative, indicating that BHA does not form DNA adducts, does not cause DNA breaks and does not interfere with DNA repair.

BHA is rapidly made bioavailable and in both rodents and humans the rate of absorption is enhanced by high-fat diets. BHA metabolism is similar in mice, rats and humans and consists of oxidative demethylation to *tert*-butylhydroquinone (TBHQ) and *tert*-butylquinone (TBQ). It is subsequently conjugated to sulfates and glucuronides and excreted predominantly in the urine. BHA enhances glutathione *S*-transferase activities in rat and mouse liver and forestomach. TBHQ forms reactive oxygen species and inhibits the prostaglandins PGE1 and PGE2.

TBHQ was tested for carcinogenicity in both $B6C3F_1$ mice and Fischer 344 rats at high dietary doses, but no carcinogenic activity was found.

BHA was given at high dietary concentrations for 13 weeks to Fischer 344 rats, followed by 1, 3, 7 or 9 weeks of BHA-free diet. The DNA labelling index and forestomach cell proliferation were increased in the high-dose groups. The DNA labelling index recovered within one week and cell proliferation subsided within nine weeks after the switch to the normal diet.

In a dose-response promotion study after initiation with *N*-methyl-*N*¢-nitro-*N*-nitrosoguanidine (MNNG), BHA at high dietary doses given during 107 weeks caused increases in the incidence of MNNG-induced tumours in the forestomach but not in the oesophagus or glandular stomach.

BHA is also known to inhibit the carcinogenic effects of genotoxic carcinogens in various tissues, including forestomach, skin, large intestine, mammary gland, liver and lymphoid tissue.

In summary, BHA induced only squamous-cell tumours of the forestomach in three rodent species. At high concentrations, the mode of action by which BHA produces forestomach tumours may involve a net production of free radicals during the long transit time in the forestomach, resulting in cytotoxicity and subsequent hyperplasia. Upon chronic administration of BHA, this hyperplasia is sustained, which leads to a tumour-promoting effect specific to the forestomach.

Catechol (see Hirose, this volume)

Catechol is a well known metabolite of benzene and is present in coal, crude wood tar, photographic developers, wood burning smoke, crude beet sugar, onions, cigarette smoke, coffee and some plant materials. It is also used in cosmetics, largely as a coupler for oxidative hair dyes.

Dietary administration of 0.8% catechol during two years is carcinogenic for the glandular stomach epithelium in male and female Fischer 344 rats, male Sprague-Dawley, Wistar and WKY rats and, although much weaker, in male and female $B6C3F_1$ mice. Weak tumorigenicity has been noted for the forestomach epithelium, with induction of papillomas particularly in male Sprague-Dawley rats. No tumours were induced by catechol in other organs. In two-stage carcinogenesis models, catechol strongly promoted rat glandular stomach and forestomach carcinogenesis after initiation with MNNG, and glandular stomach carcinogenesis in male BALB/c mice after inititation with *N*-methylnitrosourea. It also promoted cancer of the tongue and oesophagus in male Fischer 344 rats previously treated with methyl-*N*-amylnitrosamine.

Catechol is excreted in urine as sulfate or glucuronide conjugates. In addition, it can be oxidized by peroxidases to form *ortho*-benzoquinone and metabolized to 1,2,4-benzenetriol. The role of these metabolites in gastric carcinogenesis is not known.

Although catechol is not mutagenic in the *Salmonella* mutagenicity assay, it induced sister chromatid exchange, cell transformation, gene mutation, and 8-OHdG adducts *in vitro*. It is considered, nevertheless, to act as a promoter rather than an initiator in the glandular stomach epithelium, because of the lack of DNA adducts (small amounts of adducts were found in the forestomach), unscheduled DNA synthesis and DNA strand scission in this region. On the other hand, ornithine decarboxylase activity and replicative DNA synthesis were increased in the pyloric mucosa after in-vivo treatment with catechol.

In rats, catechol given in the diet induces an inflammatory response, erosion and apoptosis in the pyloric region after 12 hours, followed by increases in DNA synthesis, cell proliferation and excessive regeneration at edges of ulceration. Submucosal hyperplasia, a precursor lesion for adenomas, subsequently appears after four weeks. The results indicate that although catechol itself may have some genotoxic potential, its mode of action on the glandular stomach epithelium is non-genotoxic. Toxicity and continuous cell proliferation may thus largely be responsible for its carcinogenicity in the glandular stomach. Although the incidence of forestomach papillomas was very weak, hyperplasia of the forestomach epithelium was observed in most rats and mice treated with catechol in two-year dietary studies.

Dichlorvos (see Williams & Iatropoulos, this volume)

Dichlorvos is a contact insecticide with a fumigant and penetrant action. To date, no direct and unequivocal connection has been established between exposure to dichlorvos and cancer in humans.

When dichlorvos was given to mice in the diet, no forestomach tumours were seen, but when given by gavage it induced squamous-cell papillomas and a few carcinomas in the forestomach of female mice. In rats that received dichlorvos in the diet or by gavage a variety of tumours were observed (e.g., of the pancreas, mammary gland and leukaemias) but no forestomach tumours. An inhalation study in rats was negative.

Dichlorvos is rapidly metabolized in humans, mice and rats, by esterase-catalysed hydrolysis or oxidative *O*-demethylation. Inhalation of cholinesterase is the main biological effect of dichlorvos; in addition humoral and cell-mediated immunity is suppressed.

Dichlorvos gave positive results, without metabolic activation, in all in-vitro genotoxicity assays, but almost all in-vivo genotoxicity assays were negative.

The underlying factors operating to produce forestomach tumours in female mice treated with dichlorvos may include a combined effect of luminal conditions in the forestomach and possibly weak alkylation in lining cells, resulting from direct contact with the compound.

Ethyl acrylate (see Boorman & Sills,_this volume)

Ethyl acrylate is produced in large quantities during the production of acrylic resins, with potential for significant exposures of workers and the environment. When given by gavage to Fischer 344 rats and $B6C3F_1$ mice five times per week for 103 weeks, ethyl acrylate increased the incidence of forestomach papillomas and carcinomas combined, in rats and mice of both sexes. This treatment also increased the incidences of hyperkeratosis, hyperplasia and inflammation. Male rats treated with ethyl acrylate by gavage for six months at the high dose used in the carcinogenicity assay and then held without further treatment until 24 months of age had no forestomach tumours. Rats given ethyl acrylate for 12 months and held until 21 months showed a marginal increase in forestomach squamous-cell carcinomas and papillomas.

Ethyl acrylate given by inhalation to Fischer 344 rats and $B6C3F_1$ mice for 27 months (6 h/day, 5 days/week) did not increase the incidence of tumours at any site in either species. Only glandular hyperplasia was seen in rats.

Skin painting studies with ethyl acrylate in mice did not show any tumours. Administration of ethyl acrylate in the drinking-water during two years did not induce any treatment-related tumours in rats.

Ethyl acrylate reacts spontaneously with glutathione and protein sulfhydryl groups in many tissues. It is not mutagenic in bacteria but induces mitotic recombination in yeast, and appears to be clastogenic to mammalian cells *in vitro* but not *in vivo* (IARC, 1999). Ethyl acrylate is an example of a chemical that induces forestomach tumours in both sexes of two species of rodents when given by gavage, but only after prolonged exposure. When delivered by inhalation, in the drinking-water or by skin application, ethyl acrylate does not increase the incidence of tumours at any site.

Methyl bromide (see Boorman *et al.*, this volume)

Methyl bromide is widely used in pest control in grain storage and in fumigation of soils with potential exposure occurring both in its production and use.

Oral administration of methyl bromide in arachis oil by gavage to male Wistar rats on 5 days per week for 25 weeks was associated with severe hyperplasia of the forestomach in all treated animals, but this regressed in groups in which treatment stopped earlier. One squamous-cell carcinoma of the forestomach occurred in a rat exposed for 25 weeks. In rats of a different strain, a similar treatment for 13 weeks induced squamous-cell carcinomas of the forestomach in more than half of the animals. Methyl bromide given by inhalation to $B6C3F_1$ mice for 103 weeks, BDF_1 mice for 104 weeks or Wistar rats for 29 months failed to increase the incidence of tumours at any site. In a second inhalation study, exposure of Fischer 344/DuCrj rats to methyl bromide for 104 weeks was associated with an increased incidence of pituitary adenomas in male but not female rats, with no increased incidence of tumours at any other site.

Methyl bromide is metabolized by glutathione conjugation and excreted as carbon dioxide. It caused methylated DNA adducts *in vivo* in rats and mice. Methyl bromide is an example of a chemical that is genotoxic (see IARC, 1999) and increases the incidence of forestomach tumours in male and female rats when administered by gavage, but does not induce tumours when given by inhalation.

Sesamol (see Hirose, this volume)

Sesamol (3,4-methylenedioxyphenol, 3,4-methylenedioxy-6-hydroxybenzene) is an antioxidant found in sesame seeds and sesame oil.

When given in the diet sesamol is carcinogenic to the forestomach epithelium of male Fischer 344 rats and male and female $B6C3F_1$ mice. Hyperplasia of the forestomach epithelium occurred in virtually all treated mice and rats and, in addition, glandular stomach submucosal hyperplasia was noted in female mice. Sesamol is not carcinogenic to other organs. In a two-stage rat carcinogenesis model, dietary sesamol did not show promoting effects in any organ.

The metabolism of sesamol has not been studied in detail. The compound is not mutagenic in the *Salmonella* assay and does not form detectable DNA adducts in rat forestomach epithelium. Shortly after the start of a feeding study with sesamol, increased DNA synthesis, ulceration and regenerative cell proliferation became apparent in the forestomach epithelium of rats. Ulcers were not repaired and cell proliferation continued during treatment. When rats were administered sesamol in their diet for 24 weeks and then placed on basal diet for a further 24 weeks, atypical hyperplasia was found in areas with high DNA labelling indices. It is conceivable that during such continuous, strong cell proliferation, genetic changes in DNA could have occurred and resulted in the subsequent development of preneoplastic lesions and carcinomas.

Use of mechanistic data in the evaluation of carcinogenicity to humans

Forestomach squamous-cell tumours are most frequently induced after oral administration of a chemical, either by gavage, which results in a bolus to the forestomach, or via the diet. When given by the oral route, especially by gavage, the chemicals are more likely to give rise to forestomach tumours than when they are administered by other routes. Some carcinogens, however, can also cause forestomach tumours when exposure is by inhalation, e.g., 1,3-butadiene, for which there is considerable evidence of genotoxicity, and 2-butoxyethanol, for which there is not. After whole-body exposure to 2-butoxyethanol vapour, this chemical is present and selectively persistent in the lumen of the stomach, much of it probably resulting from the oral intake of condensed material during grooming (Green *et al.*, 2002). Chemically-induced tumorigenesis of the forestomach squamous epithelium generally appears to be a continuum, progressing from hyperplasia and dysplasia to benign tumours and eventually to malignancy. The hyperplasia induced by some agents, e.g., methyl bromide, is reversible upon cessation of exposure. For some chemicals (e.g., dichlorvos) where comparative data exist, the dependence of forestomach tumour development on administration by gavage, as opposed to exposure from food or drinking water, strongly suggests that the local concentration at the forestomach mucosa is more important than the total body dose on a mg/kg bw basis. Moreover, oral gavage with a vehicle such as corn oil results in longer retention of the agent in the forestomach than when the agent is given in water.

Various toxicodynamic factors may influence the development of forestomach tumours. Cytotoxicity and regenerative cell proliferation in the epithelium are involved in the development of forestomach tumours by many orally administered carcinogens. In the case of carcinogens that act through a genotoxic mechanism, cell proliferation may make an important contribution to tumour development. For some carcinogens not known to be genotoxic in the forestomach, irritation leading to enhanced and sustained cell proliferation may be essential for tumour development. Agents expressing cholinergic activity may prolong stomach transit time and increase reflux into the forestomach from the glandular stomach, thereby increasing the exposure of the forestomach tissue to the parent compound and/or its metabolites.

A number of multi-site carcinogens that are genotoxic appear to produce forestomach tumours through genetic alterations. For example, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) induces similar mutations in the second nucleotide of codon 13 of the c-Ha-*ras* gene in squamous-cell carcinomas of the forestomach in mice (4/7 G®T) as it does in tumours of the Zymbal gland in rats (15/15 G®T) (IARC, 1993). 1-Amino-2,4-dibromoanthraquinone induces similar H-*ras* codon 61 mutations in forestomach tumours (14/23 A®T and 6/23 A®G) as it does in lung tumours (6/16 A®T and 5/16 A®G) in mice (Hayashi *et al.*, 2001). However, the significance of these observations is not clear: forestomach tumours from mice exposed to 1,3-butadiene by inhalation contained the same H-*ras* codon 61 mutations (6/20 A®T and 3/20 A®G) (Sills *et al.*, 2001) as were found in mice treated with 1-amino-2,4-dibromoanthraquinone, which forms adducts that are probably more bulky than the hydroxy-3-butenyl-adducts of butadiene.

The precise underlying mechanism of action for any forestomach carcinogen is at present not fully known. Nevertheless, most genotoxic forestomach carcinogens appear to act through a mode of action involving genetic changes in oncogenes and tumour suppressor genes. Non-DNA reactive agents such as butylated hydroxyanisole appear to cause forestomach tumours primarily through initial cytotoxicity and subsequent sustained cell proliferation and hyperplasia.

While humans do not have a forestomach, they do have comparable squamous epithelial tissues in the oral cavity and the upper two-thirds of the oesophagus. Thus, in principle, carcinogens targeting the forestomach squamous epithelium in rodents are relevant for humans. Also, the target tissues for carcinogens may differ between experimental animals and humans and a forestomach carcinogen in rodents may target a different tissue in humans. Furthermore, tumorigenic effects in the forestomach are usually accompanied by similar effects in other tissues, indicating that there may be either general (e.g., genotoxic or receptor interactive) or multiple modes of action. However, the relevance for humans is probably limited for agents that have no demonstrable genotoxicity and that are solely carcinogenic for the forestomach squamous epithelium in rodents after oral administration, since the exposure conditions are quite different between the experimental animals and humans. Consequently, for these agents, the mode of carcinogenic action could be specific to the experimental animals.

There are considerable gaps in knowledge regarding factors that may be of importance for forestomach carcinogenesis. The role of physiological factors such as absorption and transit time on forestomach carcinogenesis is not well understood. Identification of genetic alterations in tumours induced by genotoxic and putative non-genotoxic compounds should give a clearer understanding of underlying mechanisms. Furthermore, the role of biotransformation of xenobiotics, either by the forestomach epithelium or by the luminal contents, in the induction of forestomach tumours needs to be studied on a case-by-case basis. The influence of pH on the carcinogenic process warrants attention. Also, the possible influence of neuroendocrine factors such as gastrin and other neuropeptides on the squamous epithelium of the forestomach may require further study.

In evaluating the relevance of the induction of forestomach tumours in rodents for human cancer the exposure conditions in the experiments have to be considered. The exposure conditions during oral administration are unusual (particularly if gavage dosing is employed) in that physical effects may result in high local concentrations of test substances in the forestomach and prolonged exposure of the epithelial tissue. Such factors may contribute to responses that may be unique for the forestomach. Nevertheless, carcinogens that are DNA-reactive and cause forestomach tumours in rodents — even if they only caused tumours at this site — should be evaluated as if they presented a carcinogenic hazard to humans. DNA-reactive agents with a high organ-specificity may be rare, however, because a carcinogen acting through a genotoxic mechanism would be expected to induce tumours at a number of sites. The anomaly of diethyl sulfate (for which genotoxicity has been demonstrated *in vivo*) is probably an artefact of its high chemical reactivity whereby local damage is produced and only low concentrations are available for distribution to other tissues. Agents that only produce tumours in the forestomach in rodents after prolonged treatment through non-DNA reactive mechanisms may be of less relevance to humans, since human exposure to such agents would need to surpass time-integrated dose thresholds in order to elicit the carcinogenic response. As has been summarised by Dybing & Sanner (this volume) very few of those agents that have been associated with rodent forestomach tumours are without some form of genotoxicity and approximately 85% of the total list of agents also induce tumours in other organs. The problem to be solved with any evaluation of a single agent is whether the genotoxicity is an essential property for the induction of the observed tumorigenicity. A solution to the problem is only possible after careful evaluation of all the toxicological and metabolic evidence specific to the individual agent.

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Gastric neuroendocrine tumours

Introduction

The biology of the gastric mucosa has been extensively studied in recent years, and significant progress has been made with the further elucidation of the regulation of acid secretion and with the development of agents capable of engendering substantial suppression of acid secretion. The discovery of histamine 2 receptors (H_2R) on the parietal cells responsible for the stimulation of acid secretion led to the development of the H_2R antagonist (H_2RA) class of agents for the management of acid peptic disease. Although reasonably effective in decreasing acid secretion, their mode of action — competitive receptor antagonism — culminated in tolerance and failed to provide adequate control of all forms of acid peptic disease, particularly gastro-oesophageal reflux disease and gastrinoma-induced ulceration. Following attempts to identify agents that were more effective than the H_2RAs in suppressing acid secretion, the substituted benzimidazole class of compounds (proton-pump inhibitors, PPIs) that targeted the parietal cell H^+/K^+-ATPase (proton pump) were introduced. Through covalent binding to cysteine moieties of the acid pump, these compounds block the function of these pumps and generate profound suppression of acid secretion with consequent significant clinical advantages in the treatment of acid peptic disease.

The elucidation of the mechanisms that regulate acid secretion resulted not only in the development of drugs for the treatment of acid peptic disease but also a much better understanding of both the physiology and the pathology of the gastric mucosa. Thus, the previous concept of an antral gastrin cell directly driving the parietal cell to secrete acid by a classical hormonal pathway influenced by vagal cholinergic input became significantly modified. It became evident that the target cell of gastrin in the fundic mucosa was not the parietal cell but a neuroendocrine cell — the enterochromaffin-like cell (ECL cell) — located in the lower third of the fundic gastric glands. Activation of the gastrin/CCK (cholecystokinin)-B receptor of the ECL cell by gastrin resulted in the local mucosal release of histamine that activated the parietal cell by a paracrine mechanism and resulted in H_2R activation and a complex series of intracellular events that culminated in pump activation and acid secretion. The gastrin-producing cell (G cell) was demonstrated to be under the negative regulation of luminal proton concentration (as well as the inhibitory influence of another antral neuroendocrine cell — the somatostatin cell). Of particular interest was the observation that suppression of acid secretion led not only to an increase in plasma gastrin but also to proliferation of ECL cells. Although it had long been known that gastrin exhibited a trophic effect on the gastric mucosa, in general the specificity of this activity for a particular neuroendocrine cell had not been previously recognized. Further study of this phenomenon led to the understanding that gastrin functioned not only as an ECL cell secretory agonist, but over the long term exerted a profound trophic effect on the ECL cell as well as other cellular elements of the gastric mucosa. The delineation of the interaction between altered luminal pH, whether drug-induced (by PPIs or H_2RAs) or as a consequence of disease (atrophic gastritis or pernicious anaemia) led to a further understanding of the pathophysiology of low-acid states. The gastrin hypothesis was proposed to explain the mechanism whereby decreased luminal proton concentration in the (antral or pyloric) mucosa led to increased circulating levels of gastrin and as a consequence culminated in varying degrees of fundic ECL-cell proliferation. This alteration in the neuroendocrine architecture of the fundic mucosa, although initially reversible, appeared under certain circumstances to progress through a series of advancing phases that could culminate in dysplasia and finally to cellular autonomy consistent with a neoplastic process. The precise mechanistic events responsible for this progression are as yet unclear but culminate in autonomous (gastrin-independent) growth of the ECL-cell population. Although evidence for alterations in the gastrin receptor, autocrine production of growth factors, genomic considerations and even neural regulators have been proposed to explain the mechanism, the definitive cellular events responsible for ECL-cell transformation remain to be elucidated. Initially the broad terminology of 'gastric carcinoid' was applied in a generic fashion to this neoplastic transformation. The limitations of this terminology soon became apparent since it was evident that a spectrum of neuroendocrine neoplastic diseases could exist in the gastric fundus. As a result of the advances in understanding of this pathobiology, a recent evaluation of neuroendocrine lesions of the stomach has led to the development of a more rational classification of the lesions that incorporates the cell type, pathophysiology and clinical behaviour.

As a result of the expansion in our understanding of gastric cellular physiology, knowledge of neuroendocrine tumours of the stomach has increased considerably over the last three decades. In addition, the recognition that neuroendocrine lesions are for the most part confined to the acid-secreting (oxyntic or fundic) mucosa as opposed to the gastrin-secreting (antral or pyloric) mucosa has allowed a distinction to be made between neuroendocrine tumours and adenocarcinomas.

The development of increasingly sophisticated techniques of neuroendocrine cell identification and tumour diagnosis by use of immunohistochemical probes (e.g. chromogranin, histidine decarboxylase and neuron specific enolase) has facilitated the identification of these neuroendocrine tumours. The recognition that there is a number of different endocrine cell types has helped in the understanding of the mechanisms that link the antral with the fundal components of the stomach. In this respect, description of the pivotal function of the ECL cell as the neuroendocrine regulator of parietal cell acid secretion has provided insight into the critical relationship between low-acid states, hypergastrinaemia and ECL-cell proliferation. Although the role of other endocrine cell types in gastric mucosal pathology has not been identified, it is likely that these regulatory cells are involved in the modulation of mucosal homeostasis, and the development of specific cell markers will certainly lead to their identification.

It is now evident that the neoplastic lesions of the fundic mucosa previously grouped under the generic term 'carcinoid' should be described not only in terms of the specific cell type of origin but also according to their behaviour. Thus, 'gastric carcinoid' is currently better described as an ECL-cell neuroendocrine tumour, which can be further subdivided into subtypes (Type I, II, III) depending upon the clinical behaviour of the tumour and the presence or absence of hypergastrinaemia. The question whether such lesions may alter their clinical behaviour over time or even may lead to adenocarcinoma is currently unresolved. Indeed, cell lineage and modulation of phenotype during the low-acid state, which can be induced by drugs or a variety of other chemicals are of critical interest and highly relevant in evaluating the carcinogenic risk posed by such agents.

Human gastric neuroendocrine neoplasms

Pathology and pathogenesis

The incidence of gastric neuroendocrine tumours among humans is approximately 1/1 000 000, is higher in females than in males, and shows an apparent upward trend over time (Modlin & Sandor, 1997).

Gastric neuroendocrine tumours are currently classified as *well differentiated* (with enterochromaffin-like (ECL)-cell, gastrin-producing (G) and serotonin-producing enterochromaffin (EC) cells as main components) or *poorly differentiated* (large, agressive endocrine carcinomas with poor prognosis) on the basis of the differentiation status of the majority of tumour cells (see Rindi & Solcia, this volume).

In general, a large majority of gastric neuroendocrine tumours are well differentiated neoplasms arising in the gastric fundic (oxyntic) mucosa.

Three clinico-pathologic subtypes of ECL-cell tumours have been identified and characterized: Type I ECL-cell tumours, associated with diffuse, corpus-restricted, chronic atrophic gastritis, and accounting for most of the well-differentiated neuroendocrine tumours of the stomach; they occur preferentially in aged female patients; Type II, associated with multiple endocrine neoplasia Type 1 Zollinger-Ellison syndrome (MEN 1-ZES) and hypertrophic gastropathy; these rare tumours are generally found in adults of both sexes and men; and Type III or sporadic ECL-cell tumours not associated with any distinctive gastric pathology and usually found in men in their sixties. Type I and Type II tumours exhibit the common pathogenetic condition of hypergastrinaemia, while Type III tumours are apparently independent of overt hormonal imbalances and appear to arise in a relatively normal gastric background.

Deep wall invasion and synchronous metastases are rare events in well-differentiated gastric neuroendocrine tumours. Aggressive behaviour in such a tumour is almost exclusively restricted to Type III ECL-cell tumours and is a typical feature of poorly differentiated endocrine carcinomas (PDEC).

Molecular genetic studies on gastric neuroendocrine tumours are scant. Loss of heterozygosity for microsatellite markers of the 11q13 MEN 1 locus has been identified in a number of gastric neuroendocrine tumours, and it has been reported with particularly high frequency in Type II ECL-cell tumours. Allelic loss of *p53* and *DCC* (deleted-in-colorectal-carcinoma) tumour suppressor genes were identified in both Type III tumours and PDECs. Thus significant genetic differences may exist between gastrin-dependent Type I or II and gastrin-independent Type III ECL-cell tumours and PDECs.

ECL-cell neuroendocrine tumours occur more frequently in patients with atrophic gastritis (pernicious anaemia) than previously realized (Borch, 1986). Moreover, and of great importance, the risk for these types of tumour is increased at gastrin levels > 500 pM (Sjöblom *et al.*, 1991), which is similar to the concentration of gastrin required for its maximum stimulatory effect on acid secretion (Blair *et al.*, 1987). Patients with pernicious anaemia have an increased risk not only for ECL-cell neuroendocrine tumours (Borch, 1986), but also for gastric carcinomas, which occur predominantly in the oxyntic mucosa (Elsborg & Mosbech, 1979).

N.B. - In pernicious anaemia, the most common form of vitamin B_{12} deficiency, the atrophic gastric mucosa fails to secrete intrinsic factor, which is required to transport the vitamin across the intestinal mucosa. Atrophy is usually complete when the disease is diagnosed, generally after the age of 50. It is not known when or how the process begins, nor the stages through which it passes. The pathologic process is one of atrophy without gastritis. The antrum is spared in > 80% of cases, and because there is no acid, gastrin cells increase in number and serum gastrin is high (often 1000–6000 pg/mL).

Aetiology

A major problem in the assessment of the role of anti-ulcer drugs in human epithelial gastric carcinogenesis is that the early symptoms of gastric cancer are often difficult to distinguish from those of benign peptic ulcer or gastritis. Consequently, the availability and the inappropriate use of potent anti-ulcer drugs — including H_2-receptor antagonists (H_2RAs) such as cimetidine and ranitidine, and proton-pump inhibitors such as omeprazole — may delay the diagnosis of gastric cancer (La Vecchia & Tavani, this volume).

Six analytical epidemiological studies, including nearly 1000 cases of gastric cancer, have been published on H_2RAs and gastric cancer. These studies are consistent with the absence of causal association between H_2-receptor antagonist use and gastric cancer risk. Some excess risk was apparent during the first five years of drug use, probably due to incorrect diagnosis.

Omeprazole and other proton-pump inhibitors are a different class of drug that block gastric acid secretion by altering the action of H^+/K^+-ATPase in gastric parietal cells. Although omeprazole heals peptic ulcer more rapidly than do H_2RAs, its long-term clinical efficacy on gastroduodenal ulcer is comparable to that of H_2RAs and other proton-pump inhibitors. The consequences of long-term use of proton-pump inhibitors on gastric hypoacidity are likely to be more severe than those of H_2RAs, but post-marketing surveillance data are much scantier than for H_2RAs. The latter, therefore, should be used in clinical practice when there are no specific indications to use proton-pump inhibitors, which remain the therapy of choice for Zollinger-Ellison syndrome and reflux oesophagitis.

The data described above suggest a central role for gastrin in the development of well-differentiated ECL-cell tumours of Type I and II, which together represent the vast majority of gastric neuroendocrine tumours (see Rindi & Solcia, this volume). It is far from clear, however, how the above physiological stimulus can result in ECL-cell transformation. Hypergastrinaemia appears to have little transforming effect on ECL cells of ZES patients without MEN 1 (Jensen, 1993).

Little is known about the precise mechanism of interaction with other factors, including the effects of pituitary adenylate cyclase-activating polypeptide (PACAP) and of the luminal proton concentration, and the influence of bacteria (*Helicobacter pylori*) on the regulation of ECL-cell proliferation. The neurotransmitter PACAP has been demonstrated *in vitro* to be a potent inducer of ECL-cell proliferation. Similarly it has been reported that *H. pylori* infection *in vivo* and lipopolysaccharide extracts of *H. pylori in vitro* are associated with increased ECL-cell proliferation. Thus, although it seems that gastrin plays a pivotal role in the development of ECL-cell tumours, in some circumstances factors other than gastrin may be involved. The duration of exposure may be important, and not only direct effects of gastrin may play a role but also indirect effects on an as yet unknown intermediate cell type or chemical messenger. Of particular interest is the possible underlying genomic abnormality associated with the hypergastrinaemia-related lesions occurring in conjunction with the multiple endocrine neoplasia Type I (MEN I) syndrome. Based upon currently available information, hypergastrinaemia *per se* may not be the sole agent responsible for transformation of ECL cells.

Rodent gastric neuroendocrine neoplasms

Animal models of naturally occurring gastric neuroendocrine neoplasia (see Modlin & Kidd, this volume)

Mastomys (*Praomys natalensis*) is a small, multi-mammate desert rodent species most commonly found in sub-Saharan Africa and particularly Southern Africa. Certain laboratory-bred strains of Mastomys (particularly from the Modlayo colony) are different from other mammals by their propensity for gastrin-receptor mediated ECL-cell proliferation and tumorigenesis.

Although the original distinction of the nature and origin of gastric neuroendocrine tumours in these mice was simply based upon the staining characteristics and the histochemistry of the tumour, subsequent studies have provided more sophisticated morphological and biochemical information supporting the identification of this lesion as a neoplasm of ECL-cell origin. Of further interest was the observation that the tumour secreted histamine and did not metastasize even in circumstances where the tumour size was relatively large. These characteristics are also seen in human Type I ECL-cell tumours.

The overall results of the studies with irreversible histamine-receptor blockers (loxtidine) or proton-pump inhibitors indicate that gastrin plays a critical role in promoting the rapid development of neuroendocrine-cell tumours in mastomys. However, the rate and frequency of tumour development after acid inhibition were much higher in mastomys than in other rodent species, suggesting that several factors, including hormonal and genetic effects, are important in the development of gastric neuroendocrine tumours.

An evaluation of the physiological parameters associated with regulation of acid secretion (as assessed by ^{14}C-aminopyrine accumulation in response to histamine and H_2RA inhibition) indicates that secretory regulation in mastomys is not significantly different from that of other species (rodent, rabbit, dog). Similarly, analysis of the distribution of mucosal endocrine cells has demonstrated that the density of antral gastrin cells in the mastomys is comparable to that of the rat or mouse. 5-Hydroxytryptamine-storing enterochromaffin cells, however, are considerably more numerous in the mastomys, whereas the somatostatin-producing cells of the antrum are fewer. The number of ECL cells and somatostatin-producing cells in the oxyntic mucosa of mastomys are significantly lower than in the rat and mouse.

Incidence, etiology, pathology and pathogenesis of gastric neuroendocrine tumours in rats and mice

(i) *Background* (see Chen & Håkanson, this volume)

At least five different endocrine cell types have been identified in the vertebrate oxyntic mucosa, based on their histochemical and ultrastructural features. In the oxyntic mucosa of the rat, the peptide hormone-producing cells make up about 2% of the total number of epithelial cells, and the corresponding figure in humans being 0.5–1%. The so-called ECL cells constitute 65–75% of the endocrine cells in the rat and 30–35% in man. Other endocrine cells in this tissue are the somatostatin-producing cells (5–10% in rats), A-like cells (20–30% in rats), enterochromaffin cells (in man but not in rat), and D1/P cells.

ECL cells have unique properties that serve to distinguish them from other peptide hormone-producing cells, e.g., they contain histamine and the histamine-forming enzyme histidine decarboxylase (HDC) and they function under the control of gastrin.

(ii) *Pathology* (see Hard, this volume)

Gastric tumours induced in the rat appear to originate from the base of the mucosa in the fundus, spreading through the mucosa and into the submucosa. Tumour cells of rats treated with ciprofibrate had a cytoplasm that was either foamy or pale-staining or homogeneous, faintly granular and eosinophilic. The cells with foamy cytoplasm had vesicular secretory granules with wide clear halos, which would confirm that they are derived from the ECL cell.

Microscopic lesions were identified in the gastric fundus as proliferations of neuroendocrine cells arranged in solid clusters or trabeculae. These clusters are immunohistochemically positive for cytokeratin, neuron-specific enolase, chromogranin A, and Sevier-Munger argyrophilic stain, but were negative for vimentin, muscle-specific actin and desmin. These lesions are considered to be early stages in the development of the macroscopic stomach tumours.

Gastric tumours can be locally invasive, morphologically heterogeneous neoplasms originating in the fundic mucosa and spreading to submucosa without apparent metastasis to distant organs. Cells in all macroscopic stomach tumours showed positive immunostaining for neuron-specific enolase, moderately positive staining for cytokeratin, variably positive for argyrophilic stain, negative for vimentin, and largely negative for muscle-specific actin and desmin.

(iii) *Pathogenesis*

Studies with certain chemicals have shown that the pathogenesis of gastric cancer development starts with ECL-cell hyperplasia at the base of the fundic glands, progressing through microcarcinoids to small intramucosal tumours, ultimately invading the submucosa. This progression from normal neuroendocrine cells to neoplasia is closely linked to, and dependent on, certain mucosal events of the stomach. Progressive and sustained hypergastrinaemia is pivotal. With some chemicals (e.g., butachlor and methyleugenol) other important effects include parietal cell loss and mucosal atrophy of the gastric fundus, which have never been observed with H_2RAs and proton-pump inhibitors (see Hard, this volume).

(iv) *Natural occurrence in laboratory rodents* (see Hard, this volume)

Natural occurrence of glandular stomach tumours in laboratory rodents is extremely rare; there have been no cases of gastric neuroendocrine tumours recorded in the NTP historical data bases on mouse or rat (NTP, 1998). A number of investigators using different acid-suppressing agents have demonstrated that neuroendocrine tumours in the gastric fundus of rats are discovered only after exposure to high doses of acid-suppressing drugs during nearly the entire laboratory life-span of the animals (Streett *et al.*, 1984; Ekman *et al.*, 1985; Betton & Salmon, 1986; Creutzfeldt *et al.*, 1986; Hirth *et al.*, 1988).

(v) *Etiology* (see Chen & Håkanson, this volume)

An increased incidence of gastric ECL-cell tumours has been found in rats after long-term administration of potent inhibitors of gastric secretion, including both H_2RAs (e.g., ranitidine) and proton-pump inhibitors (e.g., omeprazole) (Havu, 1986), as well as non-therapeutic compounds such as butachlor (a herbicide) and methyleugenol (a naturally occurring constituent of spices and essential oils). Other examples of chemicals inducing gastric ECL-cell tumours in rodents include ciprofibrate, loxtidine, oxmetidine, tiotidine, and omeprazole.

Additional information is provided by individual case studies of a selection of these compounds.

Butachlor (see Hard, this volume)

Butachlor is a chloroacetanilide used as a pre-emergence herbicide, in particular for weed suppression in rice paddies. Butachlor induced gastric glandular stomach tumours in rats at high dose, predominantly in females. The precursor and early neoplastic lesions show the morphological and immunohistochemical characteristics of ECL-cell neuroendocrine tumours. The butachlor-induced gastric tumours show a marked potential for local invasion into the submucosa and for becoming poorly differentiated at the cellular level. When given at the high tumour-inducing dose, butachlor causes progressive mucosal atrophy of the gastric fundus, primarily through a severe loss of parietal cells. This change is accompanied by a sustained increase in cell proliferation at the fundus base, correlating with increased proliferation of ECL cells. At the tumour-inducing dose, butachlor also produces a sustained and marked hypergastrinaemia and a substantial increase in pH of the gastric contents. Gastric neuroendocrine tumours have been observed only at the dose that produced severe mucosal atrophy (parietal cell loss), increase in gastric pH, and hypergastrinaemia. Alachlor, a chemical analogue of butachlor lacking one methyl group, has been reported to induce nasal and thyroid tumours in the rat, in addition to gastric neuroendocrine tumours. The published data on the genotoxicity of this compound are limited, but mainly negative. Butachlor has undergone extensive mutagenicity testing in in-vivo and in-vitro systems, the overall result of which indicates that it does not have genotoxic potential in mammals.

Butachlor is carcinogenic to experimental animals, causing gastric tumours of neuroendocrine cell origin in rats when administered via the diet. There is evidence that butachlor acts as a carcinogen by an indirect hormonal mechanism. The key elements of this process are progressive fundic mucosal atrophy with parietal cell loss, increasing gastric pH, progressive, severe hypergastrinaemia, and sustained ECL-cell proliferation. There is no published evidence of an association between exposure to this agent and an increased risk for cancer in humans.

Ciprofibrate (see Hard, this volume)

Ciprofibrate is a synthetic drug used in the treatment of hyperlipidaemia. It also has peroxisome proliferating activity. When administered to rats at high doses in the diet, ciprofibrate induced neuroendocrine cell hyperplasia in the gastric fundus, and after 2 years, a low incidence of invasive neuroendocrine tumours in the same region of the stomach. These cellular changes were not observed in mice or marmosets. In the early stages of exposure, ciprofibrate induced morphological changes in parietal cells of the gastric fundus in the rat. Ciprofibrate has been reported to inhibit gastric acid secretion in rats, but this could not be confirmed in subsequent studies. Several studies have shown that continuous exposure to ciprofibrate produced a sustained and marked increase in serum gastrin in rats, but at doses higher than the level at which gastric neuroendocrine tumours were induced. However, there was a correlation between the degree of hypergastrinaemia and neuroendocrine cell hyperplasia in the gastric fundus in the rat. Ciprofibrate also induced hepatocellular adenomas or carcinomas in C57BL/6N mice, a strain that is resistant to spontaneous liver tumour development. Ciprofibrate has not shown any activity in several genotoxicity assays.

When administered via the diet, ciprofibrate is carcinogenic to experimental animals, giving rise to neuroendocrine tumours of the glandular stomach in rats, and hepatocellular tumours in mice. Evidence suggests that ciprofibrate acts as a carcinogen by an indirect hormonal mechanism involving hypergastrinaemia. Ciprofibrate-induced liver tumour development is associated with peroxisome proliferation. There is no published evidence of an association between exposure to this agent and an increased risk for cancer in humans.

Methyleugenol (see Herbert & Abdo, this volume)

Methyleugenol is a natural constituent of a variety of essential oils, spices and fruit. It is primarily used as a flavouring agent in foods, and as a fragrance in cosmetics, toiletries and detergents. Methyleugenol has undergone extensive mutagenicity testing in in-vivo and in-vitro systems. While most assay results indicated a lack of mutagenic potential, methyleugenol did induce unscheduled DNA synthesis in rat hepatocyte cultures and formed DNA adducts in cultured rat and human hepatocytes, and in newborn mice and rats *in vivo*.

Methyleugenol induced neuroendocrine cell hyperplasia, and benign and malignant neuroendocrine neoplasms of the fundic glandular stomach in male and, predominantly, in female Fischer 344 rats, and marginally in male B6C3F1 mice after 2 years of oral exposure. Mucosal atrophy due to marked loss of parietal cell mass also occurred. Increased incidences of neoplasms and non-neoplastic lesions of the liver were also observed in rats and mice. Additionally, methyleugenol caused neoplasms of the kidney, skin and mammary gland and malignant mesotheliomas in rats.

Time- and dose-dependent increases in serum gastrin levels and gastric pH occurred in rats but not mice after 30 or 90 days dosing with methyleugenol, in conjunction with parietal cell necrosis (rats and mice), and mucosal atrophy and inflammation (rats). These results suggest that, at least in rats, the trophic action of gastrin may play a role in the induction of neuroendocrine cell proliferative lesions in the glandular stomach.

When given by oral gavage, methyleugenol is carcinogenic in experimental animals, based on increased incidences of gastric neuroendocrine and hepatic tumours in rats and mice and of tumours of the kidney, mammary gland, skin and mesothelium in male rats. There is evidence to indicate that a non-genotoxic mechanism contributes to the tumour-inducing effects of methyleugenol in the glandular stomach. There are no published data of an association between this agent and an increased risk for cancer in humans.

Omeprazole (see Waldum *et al.* and Chen & Håkanson, this volume)

Omeprazole and other proton-pump inhibitors block gastric secretion by altering the action of H^+/K^+-ATPase in gastric parietal cells (Maton, 1991). Although omeprazole heals peptic ulcer more rapidly than do H_2RAs, its long-term clinical efficacy on gastro-duodenal ulcer is comparable to that of H_2RAs and other proton-pump inhibitors. The latter remain the therapy of choice for the Zollinger-Ellison syndrome and are commonly used for treatment of reflux oesophagitis (gastro-oesophageal reflux disease, GORD).

In the rat, tumours developed in the oxyntic mucosa after life-long dosing with omeprazole. No other compound-related tumours were found. The gastric tumours were ECL-cell neoplasms that developed secondarily to hypergastrinaemia due to hypoacidity. It appears that omeprazole and other proton-pump inhibitors do not induce DNA damage or mutations.

In the clinical setting, patients are often exposed to proton-pump inhibitors for considerable periods of time. How ECL cells respond to this treatment and to withdrawal of the drug in humans has not been studied in any detail and not at all with respect to ECL-cell ultrastructure. However, in rats ECL cells displayed not only hypertrophy, but also cytoplasmic vacuolisation and the formation of lipofuscin bodies. The ECL-cell hypertrophy was rapidly reversed after cessation of omeprazole-induced hypergastrinaemia. Cell size decreased to below the pretreatment values within five days. A similar reversal has been observed with respect to the histidine decarboxylase (HDC) activity and the ECL-cell replication rate. In fact, the HDC activity and mitosis remained suppressed for at least 15 days after the circulating gastrin levels had been restored. After withdrawal of omeprazole, the cytoplasmic vacuoles disappeared but the lipofuscin bodies did not. The ECL-cell hyperplasia was reversible although the reversal to pre-stimulation values was slow.

The lower susceptibility of mice to develop ECL-cell growth disturbances after treatment with omeprazole compared with the rat does not mean that the mouse is less susceptible to hypergastrinaemia. The apparent species difference may only reflect that the mouse needs comparatively high doses of omeprazole to develop hypoacidity. There are recent reports on the development of hyperplastic polyps in the oxyntic gastric mucosa of humans after several years of treatment with omeprazole.

Ranitidine and loxtidine (see Modlin & Kidd, this volume)

Ranitidine is a competitive H_2RA widely used for the treatment of duodenal and gastric ulcers. It has not shown activity in a limited number of in-vitro genotoxicity tests and is considered to be a non-genotoxic compound. When administered to female Sprague-Dawley rats at high doses, sustained, elevated plasma gastrin levels, gastric mucosal hyperplasia and, after two years, the formation of benign gastric neuroendocrine tumours were observed. This drug has not been studied in other animal species, and only produces tumours in the gastric fundus of rats. Three case reports document an association between ranitidine therapy and gastrointestinal neuroendocrine tumour induction in humans, but there is no evidence from cohort studies of an increased incidence of gastric cancer among ranitidine users.

Loxtidine is a selectively acting H_2RA. It belongs to the structural class of gastric drugs that causes irreversible inactivation of this receptor. Other agents in this class (e.g., SK&F 93479 or ICI 125211) are associated with severe gastric pathology in rats after medium-term usage. SK&F 93479 induces squamous-cell tumours in the forestomach (Betton & Salmon, 1984), while ICI 125211 gives rise to malignant gastric adenocarcinomas, similar to those induced by MNNG (Streett *et al.*, 1984). None of these three drugs is available for clinical use.

Loxtidine has been extensively tested for mutagenicity. It is a non-genotoxic compound, which, when administered at high doses to rats, mice and mastomys, results in elevated plasma gastrin levels, gross gastric hyperplasia, and the formation of benign fundic neuroendocrine-cell tumours after four months (mastomys) or two years (rats and mice; a malignant neuroendocrine tumour was noted in one rat). These species differences are thought to reflect a genetic predisposition in mastomys. Tumours are limited to the gastric fundus. Loxtidine has never been clinically used, apart from an initial study evaluating its acid-suppressive effectiveness. Consequently, there are no data related to its carcinogenicity in patients.

Loxtidine is carcinogenic to experimental animals and causes fundic neuroendocrine ECL-cell tumours in rodents when given by the oral route. This effect is presumed to occur by an indirect hormonal mechanism involving hypergastrinaemia.

Mechanistic considerations (see Chen & Håkanson, this volume)

Parietal cells are the providers of gastric acidity, producing hydrochloric acid through proton-pump activation, i.e., activation of H^+/K^+-ATPase, an integral membrane protein of the parietal cell canaliculi. Gastric acid secretion is subject to feedback regulation by gastrin secretion from G cells of the diffuse neuroendrocrine system in the pylorus. As the gastric pH increases (normally as a result of food intake), gastrin production is increased, exerting in turn a trophic influence on the ECL cells in the fundus via the cholecystokinin 2 (CCK) (previously called CCK-B/gastrin) receptors, thus initiating the calcium signalling cascade. A primary effect of gastrin stimulation of the ECL cells is to increase the concentration of histidine decarboxylase, the enzyme responsible for histamine synthesis. In turn, histamine acts on the adjacent parietal cells via the H_2R, stimulating these cells to secrete acid (Håkanson *et al.*, 1986; Sundler *et al.*, 1986; Larsson *et al.*, 1988; Ryberg *et al.*, 1990; Andersson *et al.*, 1998). The marked reduction of parietal cells caused by chemicals such as butachlor provides a driving force for increased gastric pH, sustained hypergastrinaemia, and ECL-cell trophic stimulation. Sustained ECL-proliferation is known to result in the development of gastric neuroendocrine tumours in the rat (Havu, 1986; Håkanson & Sundler, 1990).

Gastrin also mobilizes mitogenic Reg-protein from the ECL cells, and this may lead to stimulated growth of the oxyntic mucosa (Fukui *et al.*, 1998). The term gastrin-ECL-cell axis has been introduced to emphasize the close functional relationship between gastrin and the ECL cells, manifested in the gastrin stimulation of ECL-cell activity and proliferation (Håkanson *et al.*, 1994; Chen *et al.*, 1999).

In view of the known stimulating effect of gastrin on ECL cells, the so-called gastrin concept was formulated to explain ECL-cell hyperplasia and tumour formation. The gastrin concept maintains that effective inhibition of gastric acid secretion abolishes luminal acid feedback inhibition of the antral gastrin cells and leads to hypergastrinaemia, which in turn activates the ECL cells, producing first accelerated mitosis and diffuse hyperplasia, then multifocal ECL-cell hyperplasia and, finally, ECL-cell tumours. According to the gastrin concept, the stimulus behind the development of ECL-cell tumours is the hypergastrinaemia rather than the achlorhydria (Håkanson & Sundler, 1990).

There is much evidence indicating that gastrin plays a pivotal role in the transformation of ECL cells regardless of species. However, it is apparent that this biological phenomenon provokes different response levels in different species. Amongst the conventional laboratory animals used in safety evaluation, the female rat appears to be the most susceptible to developing neuroendocrine ECL-cell tumours in response to chemical-induced hypergastrinaemia, and the mouse the least predictable. There are also highly susceptible rodent species, including *Mastomys* and the Japanese Cotton Rat. Hypergastrinaemia is a well-recognized trophic factor for the ECL cell, and this cell type is specifically sensitive to transformation by gastrin. There is a morphological continuum in the transformation process from ECL-cell hypertrophy to hyperplasia, dysplasia, and ultimately neoplasia, which seems to apply across species.

Detailed studies with carcinogens that induce hypergastrinaemia demonstrate that ECL cells are stimulated to increased proliferative activity within weeks of continuous exposure to the carcinogen, and this is followed shortly thereafter by a hyperplastic response. In this respect, humans may be different from other species in that several months of treatment with gastric acid inhibitors can lead to ECL hyperplasia, but as yet, this event is believed to reach a plateau and is not known to progress into neoplasia. Patients receiving H_2RAs or proton-pump inhibitors have been followed for up to 12 years with no evidence of a neoplastic risk. Given the length of time required for development of cancer in humans, often at least 20 years, the consequences of chronic exposure to these agents remain undetermined, but relative risk consistently appears to decrease with increasing exposure time in a number of epidemiological studies.

Parallels between special, highly susceptible rodent models can be drawn with certain genetic syndromes where specific gene defects are associated with high tumour responsiveness to gastric acid inhibitors. For example, sporadic cases of ZES, in which the proton concentration as well as serum gastrin are chronically increased have been followed for 20 years with no apparent development of neuroendocrine tumours. When, however, ZES is superimposed on the genetic background of multiple endocrine neoplasia (MEN 1), there is an elevated risk for ECL-cell neoplasms. Furthermore, treatment of the hyperacidity in these patients with gastric acid inhibitors predisposes to the development of ECL tumours within a relatively short time-span of four to five years. This suggests that in genetically susceptible individuals, some factor other than gastrin also plays an important role.

Use of mechanistic data in evaluation of carcinogenicity to humans

Chemicals that cause hypergastrinaemia stimulate ECL-cell proliferation in rodents and in humans.

In several species of rodents such chemicals cause neuroendocrine ECL-cell tumours of the stomach. Some of these chemicals also cause tumours at other organ sites. At least one, methyleugenol, has been shown to be DNA-reactive. Humans develop ECL-cell hyperplasia in response to H_2RAs and proton-pump inhibitors, while some conditions associated with hypergastrinaemia, including atrophic gastritis, pernicious anaemia and multiple endocrine neoplasia Type I with ZES, may increase the risk for human ECL-cell tumours. Therefore, unless there are data to indicate otherwise, ECL-cell tumours in rodents can be considered predictive of carcinogenic hazard to humans.

It was agreed that the driving factor behind neuroendocrine ECL-cell tumour development in both rodents and humans was the sustained elevation of serum gastrin, but no consensus was reached on the significance of the rat for predicting risk in man. On the one hand, it was felt that positive test results in rats were adequately predictive of human risk, while on the other hand there was concern that the high sensitivity of the rat limited the usefulness of this model for risk assessment.

Carcinogenic risk from exposure to chemicals that cause ECL-cell tumours in rodents may be increased by genetic factors and by pathological conditions such as atrophic gastritis and pernicious anaemia.

Issues to be addressed

1. Effects should be studied of combinations of agents that cause acid suppression (therapeutic drugs) and those that induce mucosal damage and are known rodent carcinogens.
2. The involvement of parietal cells, as seen with some chemical effects in rodents, in the pathogenesis of the human disease needs investigation.
3. The effect of repetitive intermittent exposure, which mimics clinical practice, should be investigated. Multiple and repetitive dosing will become more common as a result of diminishing control of the physician over the use of over-the-counter drugs.
4. The introduction of a rapid, sensitive and specific model to allow for the development of short-term risk assessment should be pursued.

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