SUBMANDIBULAR SALIVARY GLANDS AND SALIVA

AN EXPERIMENTAL STUDY IN MALE MICE ON CELLULAR GROWTH

PROEFSCHRIFT

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To my parents
sisters and brothers in law
my friends

This thesis has been prepared in the SURGICAL SERVICES, SHRINERS BURNS INSTITUTE and MASSACHUSETTS GENERAL HOSPITAL, and the DEPARTMENT of SURGERY, HARVARD MEDICAL SCHOOL, BOSTON, MASSACHUSETTS, U.S.A.

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INTRODUCTION

Cells turn over at an enormous rate in man and animals. Man loses 250 g of cells into the intestinal lumen every 24 hours (Leblond and Walker, 1956). The regulatory mechanisms of cellular proliferation have been extensively studied (for reviews see: Dowling and Riecken, 1974; Williamson, 1978a,b). Although many factors are postulated to be of influence, the exact regulatory mechanism is as yet not fully known (Lipkin, 1973; Gospodarowicz and Moran, 1976).

Studies of intestinal adaptation are increasingly relevant to modern surgical practice. First of all because recently developed techniques, including hyperalimentation, ensure the survival of many patients with massive enterocolic resections, who would formerly have died from shortage of functioning small bowel. Secondly because of the increasing numbers of enteric bypass procedures performed for morbid obesity or hyperlipidemia. In the third place because different patterns of cell proliferation may be linked with increasing susceptibilities for cancer (Cayama et al., 1978).

Factors that seem to play an important role in the regulation of cellular growth are of humoral, neural and luminal origin. There is considerable evidence that luminal factors play a dominant role in this regulation (Li et al., 1979). Of these luminal factors food, pancreaticobiliary secretions and gastric secretions have been studied most, and all of these factors seem to have an influence on intestinal adaptation (Weser et al., 1977; Williamson, 1978a,b).

Compensatory gut proliferation still occurs without gastric juice (Hughes et al., 1976; Tilson and Axtmayer, 1976; Dembrinski and Johnson, 1979) or pancreaticobiliary secretions (Shelitto et al., 1978). On the other hand, small bowel deprived of intraluminal food, no longer maintains its normal resorptive function and morphology, and causes decreased cell proliferation (Gleeson et al., 1972). This can be completely reversed by restoring intestinal continuity. Therefore, other intraluminal factors seem to be important as well, and could be produced more proximal in the gastrointestinal tract than duodenum or stomach. The hypothesis that a factor that regulates cell proliferation is secreted in saliva, is consistent with these observations.

Levi-Montalcini and Hamburger observed in 1951 that in certain mouse sarcoma cells factors were produced that stimulated neurite outgrowth in sympathetic ganglia. This factor was purified and was named Nerve Growth Factor (NGF) (Levi-Montalcini and Hamburger, 1951, 1953). Fortuitous observations demonstrated that Nerve Growth Factor was also found in high levels in snake venoms and in the submandibular salivary glands of adult male mice (Levi-Montalcini and Angeletti, 1968).

Cohen (1962) working in the laboratory of Levi-Montalcini, discovered a substance in extracts of the submandibular salivary glands of male mice that accelerated incisor eruption and eyelid opening in newborn mice. He purified this substance from extracts of the glands and called it Epidermal Growth Factor (EGF) (Cohen, 1965). Extracts of other organs, including the parotid gland of the mouse, do not possess Nerve Growth Factor or Epidermal Growth Factor. In submandibular gland saliva these growth promoting factors are found in high concentrations in adult male mice as well (Levi-Montalcini and Angeletti, 1968; Murphy et al., 1979). These glands have been shown to be rather an exocrine than an endocrine organ for NGF and EGF (Murphy et al., 1977a,b; 1979).

By in vivo and in vitro studies many potential functions of NGF and EGF have been discovered in animals. Both factors stimulate proliferation of a variety of normal and neoplastic cells in vitro (Levi-Montalcini and Angeletti, 1963; Bradshaw, 1978; Carpenter and Cohen, 1979). They can also induce ornithine decarboxylase activity, an enzyme that plays an important role in the process of cellular duplication in the gastrointestinal tract of mice (Feldman et al., 1978). Epidermal Growth Factor inhibits gastric acid secretion in rats and dogs (Bower et al., 1975) and increases the resistance of the gastromucosal barrier to ethanol in rats (Pilot et al., 1979). Recently Scheving and coworkers (1979, 1980) demonstrated that EGF promotes DNA synthesis in the exophagus, stomach, small bowel and colon in mice. Nundy et al. (1977) showed that if DNA synthesis increased, active cell prolifer-

ation occurred; this suggests that EGF promotes cell proliferation in these organs. Nerve Growth Factor can stimulate the pituitary-adrenocortical axis in rats (Otten et al., 1979).

In man Epidermal Growth Factor is found in duct cells of the submandibular gland in the Brünners glands of the duodenum (Elder et al., 1978). Urogastrone, a substance that seems to be identical to EGF, is found in urine of pregnant women and heals gastric ulcers in rats (Koffman et al., 1977). An urogastrone-like molecule was recently isolated from human serum, saliva and gastric juice (Gregory et al., 1979). High blood levels of Nerve Growth Factor are found in patients with neurofibromatosis (Fabricant et al., 1979) and in children with neuroblastoma and fibrosarcoma (Burdman and Goldstein, 1964). Moreover, high blood levels of NGF have been found in patients with Paget's disease of bone, which decrease after medical treatment (Saide et al., 1975). The meaning of these findings is not clear, but it could be that enhanced secretion of a NGF-like molecule by cells occurs when they are undergoing extensive proliferation, perhaps in the malignant status as well (Bradshaw and Young, 1976).

Since both these growth promoting factors, Nerve Growth Factor and Epidermal Growth Factor, are found in high concentrations in saliva of adult male mice (Byyni et al., 1974; Wallace and Partlow, 1976; Murphy et al., 1979) this animal seems the experimental animal of choice to define the role of these factors in vivo. High levels of NGF and EGF are only found in the submandibular glands of adult male mice, which suggests that these glands may play an important role in any cell regulation in mice through the production of saliva. Therefore we decided to study the influence of the submandibular glands in adult male mice on different kinds of cellular growth.

In the laboratory of Malt and co-workers in the Surgical Services at the Massachusetts General Hospital, Li et al (1979) showed once more in an intestinal cross gut experiment in parabiotic rats, that post resectional hyperplasia was primarily regulated by intraluminal contact. Because we had a close cooperation with the laboratory of Young and coworkers of the laboratory of Physical Biochemistry at the Massachusetts General Hospital who have extensively studied Nerve Growth Factor, we

decided to investigate the influence of submandibular saliva on cellular growth. Through the cooperation with Young and coworkers we were able to use purified Nerve Growth Factor from the submandibular gland in some experiments. However, we were not able to examine the effects of purified Epidermal Growth Factor, as this was not available.

The aim of the studies described in the present thesis was to answer the following questions.

1. What is the effect of submandibular salivary gland excision (S.G.E.) on systemic growth, metabolism and reproductive function.

2. If there is an effect of S.G.E., is this regulated mainly by humoral orintraluminal factors (i.e. saliva).

3. What is the effect of S.G.E. on stress ulcers of the stomach.

4. What is the effect of S.G.E. on wound healing.

5. What is the long term effect of S.G.E. on chemically-induced colon cancer.

CHAPTER 1

SURVEY OF THE LITERATURE

1.1. The salivary glands

Although the ancients had already described the salivary glands, they were unaware of the external secretory function (Garrett, 1975). Up till the sixteenth century it was still thought that the salivary glands were a kind of sponge which had a sieve-like role in straining off substances from the blood such as the "emunctories" (bad spirits) of the brain (Patey, 1958). In the first half of the seventeeth century saliva was yet thought to originate from the lymph (Gotfredsen, 1950). In 1656 Thomas Wharton published "Adenographia" in which he gave the first description of the duct of the submandibular gland and described then the salivary secretion. His views on the secretion were still strangely fanciful but his fundamental observation about the submandibular duct and its function opened the road to all further thinking in this field (Garrett, 1975).

Most early studies on salivary glands concerned the submandibular gland, probably due to the anatomical localisation of this gland, which is very superficial in the neck region of the animal. However in 1661 Niels Steensen described the duct of the parotid gland in a thesis and claimed that the stimulation of the gland came from the brain via the nerves, believing that these nerves had some motor function. Malpighi introduced in 1665 the word acini for the ultimate and bodies in the glands, however, general opinion still was that the glands had a kind of sieve-like role for the blood.

Another essential territory, the autonomic nervous system, was being explored but despite many hints it was not until 1850 that Carl Ludwig made a big step forward. He electrically stimulated the branch of the lingual nerve going to the submandibular gland of the dog and found that it caused a secretion of saliva from the duct of the gland. He also showed that the amount of secretion varied with the intensity

of electrical stimulation. Ludwig (1851) proved in addition that the secretory pressure could exceed the blood pressure, thus demolishing the blood filtration theory of salivary secretion. He described the sympathetic nerve to the submandibular gland. It was Claude Bernard (1879) who mapped out the peripheral pathway to the salivary glands and proved that there were two kinds of nerves with distinct origins and antagonistic actions.

Finally the introduction of the electronic microscopy disclosed the working of the myoepithelial cells and revealed that these cells often have a dual motor innervation (Garrett, 1975).

1.2. Sialadenectomy: influence in animals

Removal of all the submandibular and sublingual glands or ligation of their ducts in new born rats results in death (Plagge, 1938). If one of these four glands is left in place, life and growth continue. Death seems to occur from inanition since the young, though making attempts to suckle, fail to swallow milk (Plagge, 1938). The animals can survive by force-feeding with cow milk during the suckling period. Subsequent growth of submandibular sialadenectomized rats is retarded as compared with sham-operated controls (Bixler et al., 1955), even if pair-fed by stomach tube with the same amount of food (Shaw and Wollman, 1958; Haldi and Wynn, 1963). As the caloric value of the fecal residue between animals with and without the salivary glands is identical (Haldi and Wynn, 1963), the difference in growth is unlikely to be due to decreased food intake or impairment of absorption.

Narasimhan and Ganla (1968) found that ligation of the submandibular duct in different kinds of animals caused no growth retardation while ablation did, and suggested that the salivary glands not only had an excretory function, but had certain endocrine functions as well. The phylogenetic and embryological development of the submandibular gland moreover, is very similar to the thyroid gland (Narasimhan and Ganla, 1968). Junqueira et al. (1949) proved that ablation of the glands decreased the activity of the thyroid gland. Wase and Feng (1956b) demonstrated that the glands are capable of modifying thyroid

activity in rats and mice. This effect is not caused by interfering with the vascularity of the thyroid gland during sialadenectomy because the thyroid gland is, in the lower animals, and specially in rodents, situated at a much lower level at the neck region. For this reason, an operation at this superficial site will not interfere with the vascularity of the thyroid gland or other organs in the neck region.

As already described in the introduction, Levi-Montalcini and Hamburger discovered in 1951 Nerve Growth Factor (NGF), that has a growth promoting effect on sensory and parasympathetic ganglia in vitro and in vivo (Levi-Montalcini and Hamburger 1951, 1953). This Nerve Growth Factor was found in high concentrations only in the submandibular gland and saliva of the adult male mouse and in snake venom (Levi-Montalcini and Angeletti, 1968). Cohen discovered in 1962 another growth promoting factor in the submandibular gland and saliva of mice, that accelerated incisor eruption and eyelid opening in new-born mice. He purified this protein and named it Epidermal Growth Factor (EGF) (Cohen, 1965). Since then, many other potential functions of Nerve Growth Factor and Epidermal Growth Factor have been discovered and will be described later in this section.

Wase and Feng (1956a,b) showed that Thyroid Stimulating Hormone (TSH) reverses the growth retardation in mice after submandibular sialadenectomy. Considerable other evidence suggests that the endocrinal system is linked with the submandibular salivary glands. The adrenal glands of sialadenectomized rats are heavier and show widening of the zona glomerulosa (Bixler et al., 1955b). Sialadenectomized female mice also seem to be infertile (Hendry and Iversen, 1973). Furthermore, the submandibular salivary glands in mice exhibit sexual dimorphism, while the tubule cells of the submandibular glands undergo hyperplasia when testosterone is administered subcutaneously in castrated male or female mice (Chrêtien, 1977). These tubule cells synthesize, store and release EGF and NGF (Gresik and Barka, 1977; Simson et al., 1978). Hendry and Iversen (1973) found a marked reduction in the concentration of NGF in the plasma after removal of the submandibular glands.

The endocrine role of the growth-promoting macromolecules in the submandibular gland however, can be challenged because Byyni and co-

workers (1974) proved that ablation of the submandibular gland does not influence the plasma levels of EGF in mice. Murphy et al. (1977a) also showed that sialadenectomy did not influence the plasma level of NGF in mice, so other sites of synthesis of NGF and EGF seem likely. Some EGF is to be found in the Brünners glands of the duodenum and duct cells of submandibular gland in man (Elder et al., 1978) and NGF could be synthesized by fibroblasts in vivo since it is produced by cultured fibroblasts (Saide et al., 1975). EGF and NGF can stimulate proliferation of a variety of normal and neoplastic cells in vitro (Levi-Montalcini and Angeletti, 1963; Cohen, 1965; Turkington, 1969a,b; Bradshaw 1978; Carpenter and Cohen, 1979; Johnson and Guthrie, 1980). EGF given intravenously or subcutaneously inhibits gastric acid secretion in rats and dogs (Bower et al., 1975), increases the resistance of the gastric mucosal barrier to ethanol in rats (Pilot et al., 1979) and promotes healing of gastric ulcers in rats (Koffman et al., 1977). NGF can stimulate the pituitaryadreno cortical axis in rats (Otten et al., 1979), and like EGF it can induce ornithine decarboxylase activity in vitro in responsive cells (Greene and McGuire, 1978) and in the stomach, duodenum and colon of mice (Feldman et al., 1978). Ornithine decarboxylase in an enzyme that plays an important role in the process of cellular duplication. Since both EGF and NGF are found in high concentrations in the saliva, this suggests that these factors have an exocrine function.

The adult male mouse is the experimental animal of choice, because specially in this animal, high concentrations of EGF and NGF are found only in the submandibular gland and saliva of this gland (Cohen, 1960, 1962). Organs that have a short cell renewal time or neoplastic developments are good systems to study the growth promoting influences of saliva.

1.3. Sialadenectomy and stress ulceration

The gastrointestinal lesion which has come to be known as stress ulcer is caused by a wide variety of factors, and the pathogenetic pathways appear to be multiple. In man the incidence of stress ulcers is increasing and they complicate management of many critically ill

patients (Butterfield, 1975). To study the effects of causativ factors, one needs a good model to produce stress ulcer in the laboratory in a reproducable fashion.

During the last decades many stress ulcer models have been developed such as the restraint model, the burn model, the sepsis model, the neurogenic model and the hemorrhagic shock model. The one most widely used is the restraint model and therefore emphasis will be placed here on this model.

In the 1950s this model was developed in different laboratories. It is now thought that the ulcers produced with this model are more analogous to stress ulcers found in man than to chronic peptic ulcers in man (Lucas et al., 1971). The ulcers are located in the gastric mucosa and are multiple (Butterfield, 1975). They are superficial and, on microscopic examination, show a minimal inflammatory reaction (Bonfils et al., 1958).

The mechanism of experimental stress ulcers appears to depend on an interaction between acid, changes in mucosal circulation, an increase in the excretion of glycoproteins in the mucus, and a decrease in mitotic activity of the mucosal lining of the stomach (Butterfield, 1975). Factors enhancing ulceration are cold, starvation, increased acidity, burns, reflux of bile, endotoxin, adrenalectomy and hemorrhage. Factors inhibiting ulcerations are vagotomy, anticholinergics, elemental diets, vitamin A, antacids, prevention of bile reflux, and many others (Bonfils et al., 1960; Brodie and Hanson, 1960; Robert et al., 1979).

A good model to develop gastric stress ulcers, is starvation of the mice for 24 hours while caging them in cages with wire-mesh bottoms. Thereafter the animals are restrained by wrapping them in plaster and taping them on a rod, in such a way that there is no contact with the surrounding, and kept at 4^{o} C for 1.5 hours (Butterfield, 1975).

The effect of the submandibular gland and its secretions should be beneficial to the animal with stress ulceration because EGF, intravenously or subcutaneously given, inhibits gastric acid secretion in rats and dogs (Bower et al., 1975; Elder et al., 1978). EGF also increases the resistance of the gastric mucosal barrier to ethanol in rats (Pilot et al., 1979) and promotes healing of gastric ulcers in

rats (Koffman et al., 1977). Thus stimulation of saliva production which contains EGF might improve the healing of experimentally induced gastric stress ulcers. However, plasminogen activator, which is elevated in the blood of patients with stress ulceration, has been linked to the pathogenesis of peptic ulceration (Cox et al., 1967). Therefore submandibular gland saliva, which contains high levels of NGF, could enhance the development of gastric stress ulcers, because NGF can activate plasminogen (Orenstein et al., 1978). Since release of soluble peptides from fibrin is strictly plasminogen dependent, no detectable hydrolysis of fibrin occurs in the absence of plasminogen (Orenstein et al., 1978). Therefore is plasminogen probably important in the development of gastric ulcers.

Both EGF and NGF stimulate decarboxylase activity, an enzyme important in cell duplication, in the stomach and duodenum of mice (Feldman et al., 1978). Recently Scheving and co-workers (1979) showed a stimulatory effect of EGF on DNA synthesis in the stomach of adult male mouse.

Since many of the above mentioned effects of NGF and EGF are opposite effects to the development of gastric stress ulcers, and both these factors are found in high concentrations in saliva, we therefore decided to study the effect of sialadenectomy on stress induced gastric ulcers.

1.4. Sialadenectomy and wound healing

In normal wound healing, inflammation, granulation tissue formation, contraction and epithelialization all play a part (Edwards and Dunphy, 1958). Contraction is a major component in the closure of open skin wounds. During contraction the whole thickness of skin, including the subcutaneous layer, moves into the defect, reducing its size. This phenomenon contributes more to the wound closure in loose skin than epithelial cell migration or new connective tissue synthesis. Furthermore, contraction is independent of either epithelialization (Van Winkle, 1967) or new tissue formation (Abercrombie et al., 1960). Destruction of cells within the healing injury by

X-radiation prevents wound closure (Grillo and Potsaid, 1961).

An interesting and important question in wound healing is what mechanism triggers the chain of events leading to the wound closure. Factors as "wound hormones" or adrenal hormones are supposed to have an influence on wound healing (Van Winkle, 1967). Other investigators proposed that myofibroblasts (Gabbiani et al., 1971) or microtubules in myofibroblasts (Ehrlich et al., 1977) were involved. In a previous study we found that the epidermal matrix could play a role in wound contraction in wounds in rats (Li et al., 1980a).

Animals lick their wounds and many speculations have been made about the healing power of saliva. In saliva factors are found that could play an essential role in wound healing. Deprivation of saliva was shown to delay wound healing in rats (Shen et al., 1979) and wound contraction in mice (Hutson et al., 1979). The cleansing and moistering effects of saliva are well documented by Bibby (1949). Anti-bacterial effects on a wide variety of organisms has been found in human saliva by Van Kesteren et al. in 1942.

Experimentally-induced mouse granulation tissue displays NGF-like activity and NGF shows a kallikrein-like action in inducing contraction of isolated rat uterus (Bothwell et al., 1979), so it is possible that NGF stimulates contraction of myofibroblasts, which are believed to be responsible for wound contraction (Guber and Rudolph, 1978). EGF in saliva may stimulate epithelialization because it has a trophic effect on epithelial cells of skin (Angeletti et al., 1964). Both NGF and EGF can stimulate proliferation of a variety of normal and neoplastic cells in vitro, as mentioned in Chapter 1.2. Since these growth factors are found in high amounts in submandibular gland saliva of male mouse these glands could have an influence on wound healing. Therefore we studied the effect of sialadenectomy on wound contraction, and since we had at our disposal purified NGF we were able to study the topical effect of this protein on wounds.

1.5. Sialadenectomy and chemically-induced colon cancer

The first experimental induction of intestinal tumors was reported in 1941 by Lorenz and Stewart, who observed multiple and small in-

testinal cancers, but not colonic tumors, in mice fed dibenzanthracene or methylcholantrene. Walpole et al. (1952) produced small intestinal and colonic adenocarcinomas in a small percentage of rats, by subcutaneous injections of dimethyl-amino-biphenyl or 4-amino-biphenyl. Compounds of biphenyl-groups are actively secreted in bile, and then carried to the target cells via the fecal stream. Surgical removal of the colon from the fecal stream prevents or reduces colon tumors in rats treated with these carcinogens (Cleveland et al., 1967; Navarette and Spjut, 1967).

Laqueur (1965), searching for a possible dietary factor in amyotrophic lateral sclerosis in Guam, found that dietary factors were possibly involved in colonic carcinogenesis in mice.

Druckrey discovered in 1967 that 1,2-dimethylhydrazine selectively produced small intestinal and colonic neoplasms in inbred rats. He noted that dimethylhydrazine was structural similar to cycasin, and hypothesized that both were metabolized to methyl-azoxymethanol, and ultimately to methyl-diazonium, a potent alkylating agent (Druckrey, 1970).

Since that time, a number of chemical carcinogens have been discovered. These compounds when administered to rodents induce benign and malignant neoplasms of the colon which are strikingly similar in most respects to colon tumors in man. They provide the opportunity to study, under controlled laboratory conditions the induction of colon cancer and the effects of manipulation of different internal and external factors (diet, gut bacteria, immune system etc.) on tumor production (LaMont and O'Gorman, 1978).

Because of their chemical stability, high yield of colon cancers, and short latency period, 1,2-dimethylhydrazine and the related compound azoxymethane have become the most widely used colonic carcinogens (LaMont and O'Gorman, 1978). Dimethylhydrazine injected weekly at a dosage of 10 or 20 mg per kg of body weight produces colonic adenomas and adenocarcinomas in rats (Lingeman and Garner, 1972) and mice (Thurnherr et al., 1973); at this dosage nearly 100% of animals eventually develop one or more colon tumors, and losses attributable to acute toxicity are negligible. The latency period before development

of cancers is approximately 6 months with 10 mg per kg body weight of 1,2-dimethylhydrazine given weekly.

Wynder et al. (1969) were the first to describe a positive correlation between the amount of dietary fat and the incidence of human colon cancer. Since that time many investigators studied the influence of different intraluminal factors on dimethylhydrazine-induced colon cancers as pancreaticobiliary secretions (Chomchai et al., 1974; Williamson et al., 1978a) and dietary fiber (Burkitt, 1971; Ward et al., 1973).

Increased cell proliferation predisposes to chemical carcinogenesis (Cayama et al., 1978). Following a 50% small bowel resection, the intestinal remnant undergoes hyperplasia within 48 hours (Obertop et al., 1977). Intraluminal factors like food (Altmann, 1972; Feldman et al., 1976; Levine et al., 1976), pancreaticobiliary secretions (Williamson et al., 1978a) and duodenal chyme (Altmann and Leblond, 1970) seem to play a major role in this regulation. However, compensatory growth, following intestinal resection can still occur in their absence (Shellito et al., 1978). Li et al. (1979) proved in a cross gut experiment in parabiotic rats that regulation by intraluminal factors is more powerful than regulation by blood borne factors. Feldman et al. (1976) studied the effect of oral and intravenous feeding on the bowel remnant after a 50% proximal small bowel resection in a group of dogs. They showed a significantly increased mucosal hyperplasia and glucose absorption in the oral fed group, while the intravenously fed group showed no evidence of functional adaptation. Therefore, even if humoral factors play a necessary or permissive role, the major regulators (yet to be identified) probably work through local intraluminal contact.

Increased genetic susceptibility to colon cancer occurs in patients with familial colonic polyposis and in certain families without preexisting polyposis (Savage, 1956; Lynch et al., 1973) and in various strains of mice (Evans et al., 1974). Another factor that plays a role in the colon carcinogenesis is non-specific injury. Pozharisski (1975) fashioned a diverticulum in the cecum of rats using a pursestring suture and showed a marked increase in the incidence of cecal tumors in these animals after dimethylhydrazine treatment compared to control animals.

Rats, mice and other rodents are excellent test animals for chemically induced colon cancer because they rarely develop colorectal cancers spontaneously (Lingeman and Garner, 1972).

Recently we showed that submandibular saliva promotes intestinal proliferation (Li et al., 1980b). It is also demonstrated that submandibular sialadenectomy decreases sponge-induced granulations in rats (Teixeira et al., 1976) and slows down the growth of transplanted breast tumors in mice (Arnason et al., 1975). Since Cayama et al. (1978) showed that increased cell proliferation predisposes to chemical carcinogenesis we decided to study the effect of sialadenectomy on chemically induced colonic carcinoma in mice.

CHAPTER 2

THE EXPERIMENTAL DESIGN AND METHODS

2.1. Experimental animals

Male Swiss-strain CD mice, 40 days old and weighing 26-28 g from Charles River Breeding Laboratories (Wilmington, Ma) were generally used in the different experiments. Animals were received one week prior to the experiment for acclimatization, and were housed in cages with wire mesh bottoms to minimize coprophagia and with alternating cycles of 12 hours light and dark. The animals in the long-term chemically-induced colon cancer experiment and the animals in the growth study were housed with 5 animals together, in small cages with sawdust bedding. All mice had free access to food and water during the experiments, except where this is specially stated in the relevant chapter. Purina Chow pellets were used, containing a minimum of 4.5% fat and 23% protein. Mice were weighed weekly in the long-term projects.

2.2. Surgical procedures

All operations were performed under light ether anaesthesia between 8.00 and 13.00 hours to mitigate the effects of diurnal cycles. Specific operative techniques employed in the different experiments will be described under the relevant chapter headings, but general operative remarks are appropriate here.

2.2.1. *Anatomy of the submandibular glands*

The submandibular gland lies in a very superficial plane, just beneath the platysma, and is enclosed in a distinct capsule, which is not adherent to any fascia of the surrounding structures. The removal of the submandibular glands with the capsule will not injure the tissues such as jugular chain of lymph nodes, certainly not the thyroid gland which lies underneath the sternomastoid and hyoid muscles, while the

submandibular gland lies superficial to these structures. The blood supply of the thyroid gland is by the superior thyroid branch of the external carotid artery, which runs under the sternomastoid muscle, and the inferior thyroid artery, fro+ the thyrocervical trunk, that runs posterior to the carotid sheath. The submandibular gland is supplied mainly by the submandibular branch of the facial artery, which enters at the hilum, and a few branches of the lingual artery, as it curves round the mandible.

2.2.2. *Submandibular salivary gland excision and sham submandibular salivary gland excision*

The submandibular glands were excised through a mid-line cervical incision and the main vessels were tied with 4 - 0 silk ligatures. Care was taken to preserve sublingual and parotid glands, and not to interfere with their blood supply. Skin wounds were closed with clips, in some experiments with 3 - 0 silk. Sham-operation differed from excision, that after skin incision only full mobilization of the glands was performed.

2.2.3. *Ligation of the submandibular salivary gland duct*

Through a mid-line cervical incision the submandibular duct was ligated with 4 - 0 silk under the dissecting microscope, care being taken not to influence the blood supply to the gland. Skin closure was done with skin clips.

2.2.4. *Skin wounds in the wound healing experiment*

A 1 cm^2 rubber ink stamp was used to mark the shaven backs of animals, and the 1 cm^2 skin area, including the panniculus carnosus, was then excised. Wound contraction was measured on photographs taken with a camera having a fixed focal length and a constant magnification. The transparencies were projected onto standard graph paper, and the

tracings of the wounds were carefully cut out and weighed to the nearest 0.1 mg. Because the graph paper was of uniform thickness, the weight of the tracings represented the areas of the wounds.

2.2.5. *Stress ulceration*

Stress ulcers were produced in the stomachs of mice by withholding food, but not water, for 24 hours followed by wrapping them in plaster and suspending them from a rod in such a way that there was no contact with the surroundings. Directly thereafter they were placed at 4^{0} C for $1\frac{1}{2}$ hours. Mice were killed by cervical dislocation. Stomachs were distended gently in situ with an isotonic saline solution, removed, fixed in 10% formalin for one minute, and opened along the greater curvature. The number of mucosal defects (ulcers) in each stomach was counted under 16x magnification by an observer unaware of the pretreatment method.

If gastric instillation of substances was essential, oro-gastric intubation was performed under light ether anaesthesia, immediately before restraint.

2.3. Biochemicals

2.3.1. *Isoproterenol and bovine serum albumin*

Isoproterenol (IPR) and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, MO. The dose of IPR was 6 mg/kg, given intraperitoneally. Isoproterenol is an α-adrenergic secretagogue, that in the dosage used stimulates the submandibular gland to profuse salivation in a short period of time. Murphy et al. (1979) showed that both growth promoting factors Nerve Growth Factor and Epidermal Growth Factor are found in isoproterenol-stimulated saliva at equal levels. Bovine serum albumin served as a non-specific protein control in the gastric stress ulcer experiment.

2.3.2. *Nerve Growth Factor*

High molecular weight Nerve Growth Factor (HMW-NGF; molecular weight 116,000) was isolated from the submandibular glands of adult male mice and wis purified to homogeneity as described by Young et al. (1978). The high molecular weight form of NGF exists as a stable, oligomeric protein at the concentration found in saliva (Murphy et al., 1977). NGF is a serine protease, and its enzymic activity can be inhibited completely by diisopropyl fluorophosphate (DFP). A 2.5S subunit of NGF (based upon its sedimentation coefficient; molecular weight 26,000) is not enzymically active, but is the moiety that promotes neurite outgrowth in the sensory ganglion assay system (Levi-Montalcini and Angeletti, 1968; Bocchini and Angeletti, 1969).

The amount of saliva and NGF produced during the 1½ hours restraint in the gastric stress ulcer experiment was investigated. Saliva was collected from the pretracheal space, after transection of the submandibular ducts, in each of 10 mice after stimulation with isoproterenol. Approximately 100 µl of saliva was collected in this period in the pretracheal space. The mean NGF concentration was 70 µg/ml as estimated by the method described by Young et al. (1978a,b).

To determine the effect of this concentration of NGF on gastric mucosa, NGF was purified in the above fashion and 7 µg NGF in 100 µl of 100 mM potassium phosphate pH 7.0, was introduced into the stomachs of sialadenectomized mice. In that experiment phosphate buffer was the control solution for effects of vehicle alone. To determine whether the effect of NGF was due to the 116,000 molecular weight form of NGF, DFP-inactivated NGF and 2.5S NGF were administered intragastrically in concentrations equimolar with NGF and under identical conditions to those described above.

2.3.3. *Urokinase*

Urokinase was kindly donated by the Division of Blood Diseases and Resources, National Heart, Lung and Blood division, Bethesda, MD. Urokinase is the strongest known plasminogen activating agent.

2.3.4. *1,2-Dimethylhydrazine*

1,2-Dimethylhydrazine (DMH) was obtained from Aldrich Chemical Co. Inc., Milwaukee, WI. The doses (15 mg/kg) were administered weekly by subcutaneous injections. At this dosage nearly all animals eventuahly develop one or more colon tumors, and losses attributable to acute toxicity are negligible.

2.4. Histological specimens

At the termination of Experiment four mice were killed by cervical dislocation between 9.00 and 13.00 hours. The bowel, dissected from mesentery, was cleansed by flushing with an isotonic saline solution and the whole alimentary tract was examined under the dissecting microscope. Every suspicious area was removed and fixed in 10% formalin for microscopical examination. Specimens were coded to eliminate observer bias. Histological sections were stained with haematoxylin and eosin. All histological sections were judged by one pathologist.

2.5. Statistics

Student's t-test for unpaired data were used in most experiments. In Chapter 4 Student's t-test assuming Poisson distribution, the Chi-squared test of equality and the Fisher exact test were used. When the term significant is used in the experiments, this means statistically significant ($p < 0.05$).

CHAPTER 3

EXPERIMENT 1

DETERMINATION OF THE EFFECTS OF THE SUBMANDIBULAR GLAND AND SALIVA ON SYSTEMIC GROWTH, METABOLISM AND REPRODUCTIVE FUNCTION

3.1. Introduction

Although removal of the submandibular and sublingual salivary glands or ligation of the ducts from these glands kills newborn rats, the neonatal rats will survive and grow if they are force-fed with cow's milk (Plagge, 1938) or if vaseline is placed in the mouth to form a seal to improve suckling (Epstein et al., 1970). Beyond the obvious inference that effects of desalivation may strictly be nutritional and mechanical, there are reasons to implicate an association of the salivary glands with the endocrine system.

The retarded growth of sialadenectomized rats compared with the growth of sham-operated controls (Bixler et al., 1955a) and duct ligated animals (Narasimhan and Ganla, 1968) can be reversed by Thyroid Stimulating Hormone (Wase and Feng, 1956a). The adrenal glands of sialadenectomized adult rats are heavier than normal and display widening of the zona glomerulosa (Bixler et al, 1955b). Not only are female mice said to be infertile when sialadenectomized at maturity (Hendry and Iversen, 1973), but the submandibular salivary glands in mice exhibit sexual dimorphism, and the ductal cells of the submandibular glands undergo hyperplasia with testosterone (Chrêtien, 1977).

Moreover, growth promoting factors such as Epidermal Growth Factor (EGF) and Nerve Growth Factor (NGF) have been localized in the ductal cells of the submandibular salivary glands of the male mouse and testosterone treated female mouse (Gresik and Barka, 1977; Simson et al., 1978). Both EGF and NGF can stimulate proliferation of a wide variety of normal and neoplastic cells (Bradshaw, 1978; Carpenter and Cohen, 1979). However, ablation of the submandibular glands does not usually influence the plasma levels of EGF or NGF in mice (Byyni et al.

1974; Murphy et al., 1977). In addition, because both EGF and NGF are found in high concentrations in the saliva (Byyni et al., 1974; Wallace and Partlow, 1976; Burton et al., 1978), this suggests that these factors have an exocrine function.

To define directly some of the endocrine and exocrine functions of submandibular glands in mice, we studied the effects of submandibular sialadenectomy and duct ligation on growth and metabolic rate in male mice and the effects of submandibular sialadenectomy on the reproductive function in both male and female mice.

3.2. Materials and methods

In the study on systemic growth, 21-days old male Swiss mice (n=60; 9-12 g) were randomly divided into 3 groups. Twenty-one days is the youngest age that the mice can be weaned from the mother. From the first group, the submandibular salivary glands were excised as described in Chapter 2.2.2. From the second group, the submandibular ducts were ligated, care being taken to preserve the blood supply to the submandibular glands. In a third group a sham operation was carried out.

For the first six weeks after operation, the average daily food consumption of mice in each group was assessed. The amount of standard Purina rat Chow pellets presented daily to each group was weighed. The amount of food left, including crumbs dropped through the mesh bottom was collected the next day and measured, so the differences in the amount of food presented and recovered indicated the amount of food consumed.

All the mice were weighed weekly for 32 weeks. At the age of 10 weeks, 20 weeks and 30 weeks, the volumes of oxygen consumed (VO_2) and of carbon dioxide expired (VCO_2) by each animal were determined. The metabolic apparatus to study this consisted of an air-tight, temperature-regulated (24^{o} C) metabolic chamber that was part of a closed system including a volumeter (model 160, Med-Science Electronics, St. Louis, MO) and a roller pump that provided a rapid turnover of air (Fig. 1).

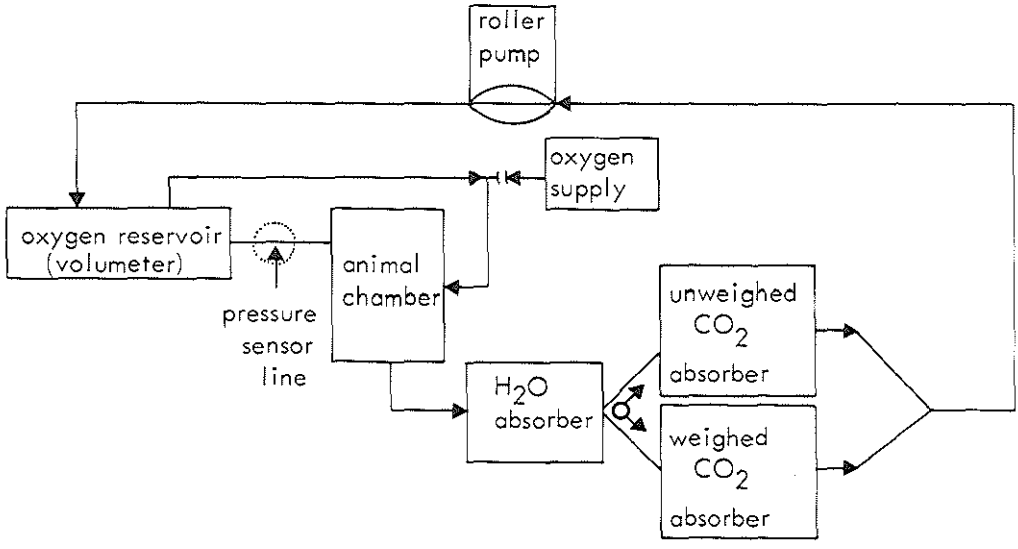

Figure 1. The metabolic apparatus with direction of air flow in the metabolic study.

While a mouse was being accustomed to the air-tight metabolic chamber, including the noise of the roller pump which provided the circulation of air, water vapor and carbon dioxide in the circuit were removed through a water absorber (Drierite) and a unweighed CO_2 absorber (Ascarite). The circuit and the oxygen reservoir were then filled with oxygen. During a standard period of 12 minutes the circuit was switched over from the unweighed CO_2 absorber to a weighed CO_2 absorber without venting the system to the atmosphere. The expired CO_2 and water thus were trapped, and the O_2 consumed was replaced by an equal amount of O_2 entering the system from the volumeter. The VO_2 was calculated from the rate at which the volumeter emptied; VCO_2 was determined from the weight change in the CO_2 absorber.
The formulas used were:

$$VO_2 = \frac{\text{distance (cm) moved by pressor sensor line in } O_2 \text{ reservoir} \times 5.1 \times 0.92}{\text{weight of animal (g)} \times 0.0224 \times t \text{ (mins.)}}$$

where 5.1 = conversion factor to ml of O_2
0.92 = conversion factor to STPD ml O_2
0.0224 = R constant
time = 12 minutes (our standard period)

$$VCO_2 = \frac{\text{weight change (g) due to } CO_2 \text{ absorption} \times 509}{\text{weight animal (g)} \times 0.0224 \times \text{time (mins)}}$$

where 509 = conversion factor from g CO_2 to ml STPD CO_2

$$\text{Respiratory quotient (RQ)} = \frac{VCO_2}{VO_2}$$

In the fertility study, 21 days old male (N=16) and female (n=16) mice (9-12 g) were used. Half the animals of each sex were subjected to submandibular salivary gland excision, the others underwent a sham procedure. Post operatively, the animals were housed in pairs, one male with one female, providing 4 groups of each 4 pairs of animals. The groups were formed as described in Table I. The female mice were examined daily for vaginal plug as evidence of mating and were weighed daily. The length

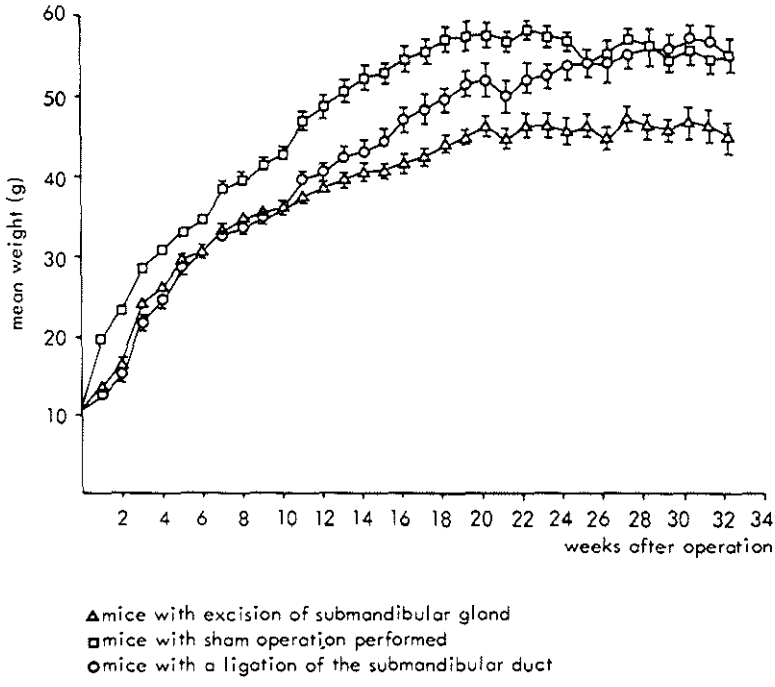

Figure 2. Curves of weekly weights of mice in growth study.
All groups contained 20 animals.
Values are means ± S.E.M.
From second week till end of the experiment weight-differences of S.G.E.-group from sham-operated animals significant at $p < 0.001$.
From second week till fifteenth week of the experiment weight-differences of duct ligated group from sham-operated animals significant at $p < 0.001$.

of gestation period, the number of mice in each litter and their weights were recorded in the pairs of mice in each group.

3.3. Results

3.3.1. *Growth study*

In the first 3 weeks after operation one animal died in the salivary gland excision group and four animals in the duct ligated group. No animal died in the sham group. All three groups ate similar amounts of food daily in the first six weeks after operation. The daily intake of food for each group is seen in Table 2, no statistically significant differences were found.

Despite similar food intake, at six weeks after operation, the weight of the sham-operated animals was 12% greater than those with duct ligation ($p < 0.001$) and 13% greater than those with submandibular gland ablation ($p < 0.001$). Throughout the experimental period of 32 weeks, the sham-operated animals were consistently heavier than the mice without submandibular glands. However the weights of the duct ligated mice were initially similar to those of the sialadenectomized mice up to 15 weeks after operation. By 22 weeks after operation, the weights of the duct ligated mice caught up with that of the sham-operated animals so that the duct ligated mice were heavier than the sialadenectomized mice and had a similar weight to the sham group at that time (Fig. 2). At any time point after the second week of the experiment, the weight difference between the sham-operated group and the salivary gland excision group was significant at $p < 0.001$ (Fig. 2, Table 3). During the first 10 weeks after operation the weight difference between the sham group and the duct ligation group was significant at $p < 0.001$, in the period 10-16 weeks after operation the significance was at $p < 0.01$ (Fig. 2, Table 3). Eighteen weeks and later after operation there was no statistically significant weight difference anymore, between the sham-operated animals and the duct ligation group.

Table 1. GROUPS FORMED IN PREGNANCY STUDY

	Male	Female
Group I	SHAM (4)	SHAM (4)
Group II	SGE (4)	SGE (4)
Group III	SGE (4)	SHAM (4)
Group IV	SHAM (4)	SGE (4)

SGE = mice with salivary gland excision
SHAM = mice with sham operation
( ) = number of animals

Table 2. MEAN DAILY FOOD INTAKE DURING THE FIRST SIX WEEKS OF THE EXPERIMENT PER ANIMAL

SHAM group	5,2 g ± 1,2 g*	(mean ± S.E.M.)
SGE group	5,4 g ± 1,0 g*	(mean ± S.E.M.)
DUCT LIGATED group	4,8 g ± 1,1 g*	(mean ± S.E.M.)

SHAM group = sham sialadenectomized animals
SGE group = submandibular salivary gland excision animals
DUCT LIGATED group = animals with a ligation of the submandibular salivary gland duct

*None of these differences are significantly different from each other

Table 3. WEIGHT OF ANIMALS AT DIFFERENT TIMES DURING THE EXPERIMENT (MEAN ± S.E.M.)

	SHAM	SGE	DUCT LIGATION
Operation day	11.4 ± 0.7 g	11.2 ± 0.8 g	11.0 ± 1.1 g
4 weeks after operation	30.7 ± 2.4	26.4 ± 2.8*	24.8 ± 4.0*
8 weeks after operation	39.8 ± 4.5	33.8 ± 3.6*	35.3 ± 3.5*
12 weeks after operation	49.0 ± 7.5	39.4 ± 3.8*	42.4 ± 5.6**
18 weeks after operation	57.6 ± 8.7	44.4 ± 6.5*	51.1 ± 8.6*
26 weeks after operation	55.8 ± 9.1	45.5 ± 7.6***	54.6 ± 9.0
32 weeks after operation	53.7 ± 10.5	45.5 ± 8.5***	56.0 ± 10.0

* difference from sham-operated group significant at $p < 0.001$

** difference from sham-operated group significant at $p < 0.01$

***difference from duct-ligated group significant at $p < 0.01$

3.3.2. *Metabolic study*

Metabolically, the sialadenectomized and duct-ligated mice behaved similarly but differed from the sham group in all three intervals studied (Table 4). Although the VO_2 of the three groups was nearly the same, the respiratory quotient of the sham group was much higher than that of the two groups ($p < 0.01$) at 10, 20 and 30 weeks. The respiratory quotient of mice lacking submandibular saliva indicated primarily utilization of fat as an energy substrate.

3.3.3. *Pregnancy study*

In this study, three out of four female mice in each group became pregnant and gave birth to live litters. There was no statistically significant difference between the sizes of the litters or their weights (Table 5).

Table 4. OXYGEN CONSUMPTION, CARBON DIOXIDE OUTPUT, AND RESPIRATORY QUOTIENT IN THE METABOLIC STUDY

TIME	SHAM group	SGE group	DUCT LIGATION group
10 weeks			
VO_2 (μmole/kg/min ± SEM)	1.677 ± 0.057	1.592 ± 0.062	1.417 ± 0.050
VCO_2(μmole/kg/min ± SEM)	1.494 ± 0.040	1.126 ± 0.058	1.016 ± 0.051
VCO_2/VO_2	0.891 ± 0.026*	0.707 ± 0.012	0.717 ± 0.011
20 weeks			
VO_2 (μmole/kg/min ± SEM)	1.341 ± 0.033	1.471 ± 0.063	1.310 ± 0.024
VCO_2(μmole/kg/min ± SEM)	1.177 ± 0.028	1.044 ± 0.076	0.964 ± 0.038
VCO_2/VO_2	0.876 ± 0.023*	0.710 ± 0.013	0.736 ± 0.011
30 weeks			
VO_2 (μmole/kg/min ± SEM)	1.451 ± 0.080	1.439 ± 0.061	1.213 ± 0.075
VCO_2(μmole/kg/min ± SEM)	1.220 ± 0.053	1.022 ± 0.041	0.910 ± 0.063
VCO_2/VO_2	0.860 ± 0.028*	0.713 ± 0.011	0.755 ± 0.010

*Differences in the respiratory quotient of the sham-operated group from the other two groups significant at $p < 0.01$

Values are means ± S.E.M.

Table 5. RESULTS OF PREGNANCY STUDY

	PREGNANT (%)	MICE PER LITTER (mean ± SEM)	WEIGHT OF MICE PER LITTER g (mean ± SEM)
Group I	75	7.0 ± 0.58	1.663 ± 0.020
Group II	75	8.3 ± 1.5	1.659 ± 0.017
Group III	75	7.7 ± 1.2	1.671 ± 0.017
Group IV	75	7.7 ± 0.9	1.695 ± 0.015

Differences are not significant. See for Groups Table 1.

3.4. Discussion

Our finding that removal of the submandibular salivary glands in male mice retarded systemic growth is in agreement with other reports, though previously rats were used and the salivary glands removed were not specified (Shaw and Wollman, 1958; Haldi and Wynn, 1963).

In our study deprivation of submandibular saliva in male mice by ligation of the submandibular duct also produced immediate growth retardation similar to that following submandibular sialadenectomy; this contrast to the previous observations that duct ligation in pre-weaned mice had no effect on growth when followed for one month after operation (Narasimhan and Ganla, 1968). The weight gain of submandibular duct-ligated animals was the same as submandibular sialadenectomized mice up to 15 weeks after operation. Although weight differences can occur in rats with similar food intake, but fed for 3 hours daily as compared with rats fed ad libitum (Callaghan, 1979), the observed weight difference in our mice cannot be explained by a restricted feeding schedule because all the mice had free access to food at all times.

We observed no difference in the food intake between the three groups of mice despite the difference in systemic growth. Animals with and without salivary glands pair fed by stomach tube with the same amount of food have previously been shown to have different growth rates despite identical fecal caloric contents (Haldi and Wynn, 1963). Therefore, the differences in growth are unlikely to be due to decreased food intake or impairment of absorption, but rather to a metabolic derangement, which in other reports, can be reversed with Thyroid Stimulating Hormone (Wase and Feng, 1956a). Moreover we have shown that animals deprived of submandibular saliva, whether by gland excision or duct ligation, have different respiratory quotients to the control animals despite similar food intake. The respiratory quotient of the control animals indicated metabolism of a mixed diet while animals lacking in submandibular saliva were primarily utilizing fat. This change in metabolism agrees with the finding of Haldi and Wynn (1963), that only less body fat was found in sialadenectomized animals with no other

alteration of body constituents. Moreover, even after the catch-up in weight with the control animals, the duct ligated mice behaved metabolically in the same fashion as the sialadenectomized animals, thereby suggesting a role for submandibular saliva in regulating metabolism.

We do not have a definite explanation for the catch-up in the duct ligated animals after 15 weeks. The only weak explanation till now is that Emmelin et al. (1974) demonstrated after duct ligation in cats for 81 days, that 1% of the saliva still may escape. There might escape more saliva in mice after duct ligation. This could account for the weight gain in the duct ligated animals over a prolonged period of five to seven months in our study. However, this small amount of saliva is unlikely to compensate for the metabolic alteration so that this derangement persists.

We were not able to confirm the observation of Hendry and Iversen (1973) that submandibular sialadenectomized female mice became infertile, because in our study submandibular sialadenectomy in either male or female mice had no effect on the fertility rate or the subsequent outcome of the pregnancy. Therefore, although the submandibular ductal cells are stimulated by testosterone (Chrêtien, 1977), they do not appear to have an influence on the sexual reproductive function.

The effects of the submandibular salivary glands on growth and metabolism are likely to be mediated by the growth promoting factors such as Epidermal Growth Factor and Nerve Growth Factor, produced by the ductal cells and carried in high concentrations in the saliva. An additional endocrine function of the submandibular gland, mediated entirely by the blood stream, cannot be confirmed because ligation of the submandibular duct will alter the metabolism of the animal and causes immediate growth retardation to the same extent as submandibular sialadenectomy.

CHAPTER 4

EXPERIMENT 2

DETERMINATION OF THE ROLE OF THE SUBMANDIBULAR GLAND AND SALIVA IN GASTRIC STRESS ULCERATION

4.1. Introduction

Although the pathophysiology of stress ulcers, is still not clear, it is generally agreed that gastric acid secretion plays an important role in the disease (Butterfield, 1975; Weser, 1978; Dyck, 1979).

The clinical observation of the very low incidence of peptic ulceration during pregnancy, led to the discovery that extracts of the urine of pregnant women had a beneficial effect on experimental stress ulcers in dogs (Sandweiss et al., 1938). Gregory isolated the structure in urine responsible for the decreased gastric acid secretion (urogastrone) and demonstrated later that this urogastrone was identical to Epidermal Growth Factor (EGF) (Gregory, 1975). The data of Cohen (1965) indicated that EGF accelerated incisor eruption and eyelid opening in new born mice; in vitro EGF stimulated a variety of normal and neoplastic cells (Cohen, 1965; Carpenter and Cohen, 1979). Pilot et al. (1979) demonstrated recently that EGF increases the resistance of the gastric mucosal barrier to ethanol in rats.

EGF is found in high concentration in the submandibular salivary gland of male mice (Cohen, 1965). Nerve Growth Factor (NGF) is also found in high concentrations in this gland (Levi-Montalcini and Angeletti, 1963). It has been shown that NGF induces ornithine decarboxylase activity, an enzyme essential for cell duplication, in the stomachs and duodenums of mice (Feldman et al., 1978); NGF also can stimulate proliferation of a variety of normal and neoplastic cells in vitro (Bradshaw, 1978).

Since both growth factors EGF and NGF are found in high concentrations in submandibular gland saliva of adult male mice (Murphy et al., 1977) we decided to study the effect of submandibular saliva on stress-induced gastric ulcers in male mice.

4.2. Materials and methods

Experimental gastric stress ulcers were produced in mice with and without the submandibular glands. Operations were performed and stress caused as previously described in Chapter 2. Animals were all Swiss-strain male mice 40 days old, weighing 26-28 g.

In the first part of the study a sham-operated group (n=11) was compared with an excision group (n=12). In both groups the stress procedure was started immediately after operation. In the second part of the study a sham-operated group (n=11) was compared with an excision group (n=12). In these groups the stress procedure was started one week after operation, to exclude the stress-influence of the operation itself. Since these two experiments showed that there was no difference in the effects if the restraint started immediately after operation or one week after operation, we decided to start the restraint in the rest of the study directly after operation (see Table 1).

In the third part of the study we compared a sham group (n=13) with an excision group (n=11). In this study we stimulated the saliva production of the animals with isoproterenol (IPR) in a single intraperitoneal dosage of 6 mg/kg, given immediately before the restraint period.

Because EGF contained in the saliva seems to protect against stress ulceration (Bower et al., 1975; Young et al., 1978a; Pilot et al., 1979) we decided to investigate the action of HMW-NGF. An amount of HMW-NGF, equimolar to that produced in submandibular gland saliva during 1.5 hours after isoproterenol stimulation and duct transection, was introduced into the stomachs of sialadenectomized male mice (n=40) immediately before restraint, via an orogastric catheter. As control solution for effects of vehicle alone we used 100 mM potassium phosphate buffer pH 7.0, introduced in the stomachs of sialadenectomized mice (n=34) in the same way and at the same time during the stress schedule. To determine whether the effects were a result of the enzymically active 116,000 molecular weight form of NGF, DFP-inactivated HMW-NGF and 2.5 S NGF were administered intragastrically in concentrations equimolar to HMW-NGF under conditions identical to those described above in respectively 28

and 13 sialadenectomized mice. To see whether the effects were due to plasminogen activation of HMW-NGF, equimolar amounts of urokinase were instilled into the stomachs of 22 sialadenectomized animals. Finally, to exclude a non-specific protein effect of HMW-NGF, bovine serum albumin was administered in the same way into the stomachs of 14 sialadenectomized male mice.

4.3. Results

About 4 ulcers per stomach were noted in both control and sialadenectomized mice whether an operation was performed 1 day or 1 week before stress (Table 1). The number of gastric lesions in mice with intact submandibular glands nearly doubled after treatment with isoproterenol (Table 1). HMW-NGF aggravated stress ulceration in animals without submandibular glands to an extent similar to that produced by IPR stimulation in control mice (Table 2). The number of stress ulcers in sialadenectomized animals treated with HMW-NGF was significant higher than in sialadenectomized animals treated with phosphate buffer, bovine serum albumin, 2.5 S NGF, NGF inactivated by DFP and urokinase (Table 2). All the treatments other than sham-operation plus IPR or administration of HMW-NGF caused about 4 ulcers per stomach (Table 1 and 2).

4.4. Discussion

Stimulation of saliva production nearly doubled the number of gastric stress ulcers, which developed during 1.5 hours of restraint. The reason that this only happened after saliva stimulation could be that the non-stimulated saliva production is too small in 1.5 hours to show an effect. The results of our experiment also indicates that instillation of 7 μg NGF (the amount of NGF produced in the stimulated submandibular salivary glands in 1.5 hours) by orogastric tube in sialadenectomized mice likewise doubled the number of gastric stress ulcers in these animals.

The results of this experiment were unexpected. Since Epidermal Growth Factor (EGF) inhibits gastric acid secretion (Bower et al. 1975),

Table 1. EFFECTS OF SIALADENECTOMY ON STRESS ULCERATION

TREATMENT	STRESS ULCERS PER STOMACH (MEAN ± S.E.M.)	
	Operation and immediate stress	Operation and stress one week later
Sham sialadenectomy	4.2 ± 0.7 (11)*	4.4 ± 0.7 (11)
Sialadenectomy	3.7 ± 0.7 (12)	4.0 ± 0.7 (12)
Sham sialadenectomy + IPR	7.7 ± 0.8** (13)	
Sialadenectomy + IPR	4.6 ± 0.7 (11)	

* ( ) = number of mice

**p <0.01 compared with mice not stimulated by IPR and p <0.001 compared with the sialadenectomized mice stimulated by IPR.

IPR = Isoproterenol

Table 2. STRESS ULCERS IN SIALADENECTOMIZED MICE AFTER OROGASTRIC INTRODUCTION OF DIFFERENT SUBSTANCES

TREATMENT	STRESS ULCERS PER STOMACH (MEAN ± S.E.M.)
HMW-NGF	7.0 ± 0.4** (40)*
Phosphate buffer	4.1 ± 0.4 (34)
Bovine serum albumin	3.4 ± 0.7 (14)
2.5 S NGF	4.7 ± 0.9 (13)
NGF inactivated by DFP	4.0 ± 0.4 (28)
Urokinase	4.9 ± 0.6 (22)

* ( ) = number of mice

**p <0.001 compared with buffer, bovine serum albumin, and DFP-inactivated NGF groups and p <0.01 compared with the urokinase and 2.5 S NGF groups.

DFP = Diisopropyl phosphofluoridate

increases the resistance of the gastric mucosal barrier to ethanol (Pilot et al., 1979) and promotes healing of gastric ulcers (Koffman et al., 1977), we expected that submandibular saliva, which contains EGF in high concentrations, woule have a beneficial effect on gastric ulcer formation. Moreover, Nerve Growth Factor (NGF) is known to stimulate neurite outgrowth, while NGF and EGF stimulate cellular proliferation in vitro in a variety of normal and neoplastic cells (Bradshaw, 1978; Carpenter and Cohen, 1979). Both factors induce ornithine decarboxylase activity, an enzyme essential for cell duplication, in the stomachs and duodenums of mice (Feldman et al., 1978; Nagaiah et al., 1978).

Elevated plasminogen activator, present in the blood of patients with stress ulceration, has been linked to the pathogenesis of peptic ulceration (Cox et al., 1967). Since NGF can activate plasminogen (Orenstein et al., 1978) this could be the way in which saliva and NGF aggravates stress ulceration. However, we found that gastric instillation of urokinase, the strongest known plasminogen activating agent, had no effect on the number of stress ulcers. Therefore we have to conclude that it is not the plasminogen activating property of NGF that is responsible for the doubling of number of stress ulcers in the present experiment.

Otten et al. (1979) recently showed that systemic administration of NGF stimulates hypothalamo-pituitary-adrenal axis. Murphy and coworkers (1979) also demonstrated that hippocampal lesions that cause high plasma corticosterone levels in rats, combined with restraint, markedly increase ulcer formation. The explanation of the results found in our study could be that Nerve Growth Factor acts as stimulator of the pituitary-adrenocortical axis, and the high levels of corticosterone combined with the restraint results in a greater number of gastric ulcers.

CHAPTER 5

EXPERIMENT 3

DETERMINATION OF THE EFFECTS OF SALIVA ON WOUND HEALING

5.1. Introduction

In the animal world it is well known that animals lick their wounds. The cleansing, moistering, and antibacterial functions of saliva have been adequately documented (Van Kesteren et al., 1942; Bibby, 1949). Wounds protected from bacterial contact heal more slowly and have a lower tensile strength than those exposed to bacteria or mild physical irritation (Shafer et al., 1974). On the other hand, a wound massively infected by bacteria will heal slowly, or not at all (Shafer et al., 1974). Shen et al. (1979) experimented with sialadenectomized rats and showed, comparing these animals to sham-controls, that there was no influence on the wound healing of a tongue wound (healing by first intention), however the restoration of gingiva was significantly delayed after wounding.

In normal wound healing, inflammation, granulation tissue formation, contraction, and epithelialization all play a part (Edwards and Dunphy, 1958). Wound contraction is a major component in the closure of open skin wounds, believed to be caused by myofibroblasts (Guber and Rudolph, 1978). Hutson et al. (1979) showed that sialadenectomy or duct ligation retarded wound contraction in female mice, and suggested that the mouse submandibular glands contained a factor or factors largely applied by licking, which accelerated early wound contraction.

Many studies showed that Nerve Growth Factor and Epidermal Growth Factor are secreted in high concentrations in saliva of mice (Wallace and Partlow, 1976; Murphy et al., 1977). We showed an effect of saliva of mice on systemic growth (see Chapter 3) and an effect of mice saliva on stress ulcers (see Chapter 4). Therefore we decided to repeat a part of the experimental work of Hutson and co-workers (1979),

to study the effect of submandibular salivary gland excision on wound healing in mice; however in our experiment we studied male mice, because saliva of these animals contain higher levels of NGF and EGF than female mice. Since we speculited that these growth factors might play some biological role in the process of wound healing, we studied also the effect of topical application of NGF on wounds in sialadenectomized mice. We could not applicate EGF because this was not available.

5.2. Materials and methods

40 Days old Swiss strain male mice, weighing 26-28 g were used for this study (n=188). All animals underwent a sham sialadenectomy or a submandibular salivary gland excision as described in Chapter 2. Wounds were made in the center of the back of each animal, in such a way that they were unable to lick their own wounds (see Chapter 2). All wounds were made under light ether anesthesia, immediately after sialadenectomy or sham-operation. Sham-operated animals received slightly less food to ensure equal body weights between the groups. They had free access to water and a 12 hours lighting cycle was maintained. Photography at fixed focal length was used to measure wound areas on alternate days as described in Chapter 2. The color transparencies of the wounds were projected onto uniform weight paper, the wound areas traced and these were cut out and weighed to the nearest 0.1 mg.

In the first part of the experiment, in which we explored the effect of sialadenectomy versus sham-operation, we divided the animals into three groups. The first group of sham-operated control animals (n=25) were housed together in one cage. The second group of sialadenectomized animals (n=25) were all together placed in a separate cage, and a third group of animals (n=18), half of which were sham-operated and half sialadenectomized, were placed in a third cage (mixed group).

To study the effect of topically applied reagents we sialadenectomized a total of 120 mice and divided them into six groups; the six groups were separately caged. So in the study of topical applicated

reagents all mice had a submandibular salivary gland excision. HMW-NGF was purified from submandibular glands of adult male mice in the way described by Young et al. (1978a) (see also Chapter 2). In one group of animals this was topically applied to the wound area immediately after injury, and thrice at 12 hours intervals.

After we showed that HMW-NGF had a topical effect on the wound contraction (see Results) we decided to try to determine which part of the NGF-molecule was responsible for this wound contraction. Therefore we applied inactivated HMW-NGF (this is the HMW-NGF molecule, without enzyme activity), trypsin (to detect if the esterase activity of NGF was responsible), urokinase (to detect if the plasminogen activating property of NGF was responsible) or 0.1 mM phosphate buffer (to exclude that the vehicle was responsible) to the wounds of five groups of sialadenectomized animals. All solutions were given in 0.1 mM potassium phosphate buffer at pH 7.0. The treatment schedule for each reagent consisted of topical application to the wound area immediately after injury and three additional applications at 12 hours intervals, so that total treatment was restricted to a 36 hours period.

5.3. Results

No animal died during the experiment. All groups of animals gained weight, and there were no statistically significant differences between the weights of the mice in each group during the experiment. During the whole experiment we observed that the mice were licking each others wounds. Figure 1 represents the percentage of the initial wound areas as a function of time after injury for the mice with a sham operation and the animals with a submandibular salivary gland excision. The wound area contracted significantly faster in the animals with submandibular salivary glands in situ ($p < 0.001$), up to 8 days after injury. When sham-operated controls and sialadenectomized animals were housed together in the same cage (mixed group), the rates of wound closure were not significantly different at any time point from those of mice with intact submandibular glands (Table 1). The difference in wound closure compared to animals with sialaden-

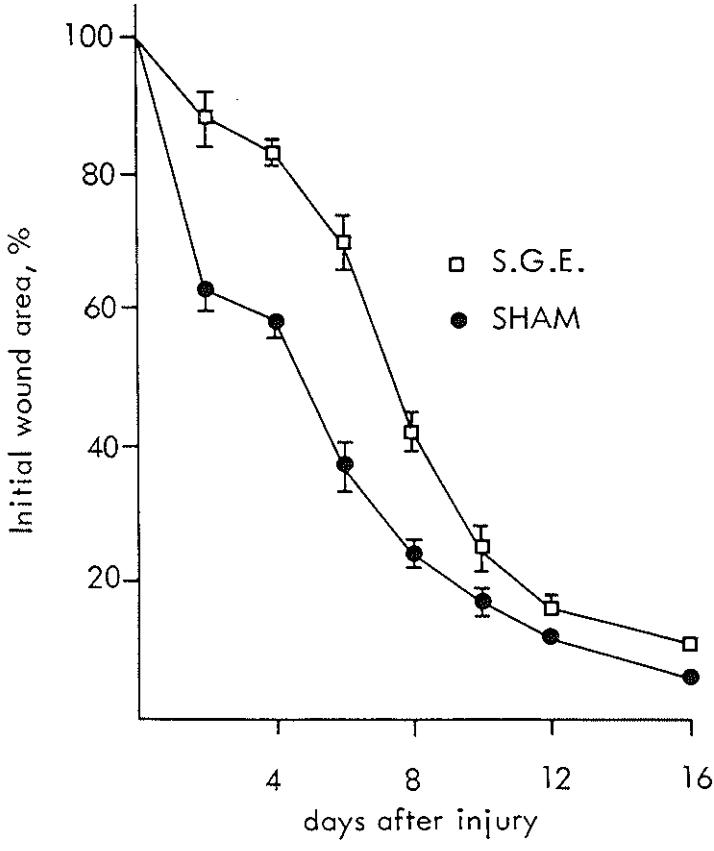

Figure 1.
Effects of sialadenectomy or sham-sialadenectomy upon rates of wound contraction.
Each time point represents 25 wounds.
Values are means ± S.E.M.
Up to day 8 after operation differences are significant at $p < 0.001$.

ectomy however, was significant ($p < 0.001$).

Figure 2 shows the degree of wound contraction in the sham group, the sialadenectomized animals and the group of animals in which HMW-NGF was applied to the wounds during a 36 hours period. The degree of wound contraction in the NGF treated group was similar to that of sham operated animals up to day 4, and it differed markedly from wound areas of sialadenectomized animals ($p < 0.001$). Comparison of wound contraction between HMW-NGF treated wounds to either phosphate buffer, 2.5 S NGF, DFP-inactivated NGF, trypsin, or urokinase treated wounds showed significant difference up to day 4 ($p < 0.001$) (Fig. 3, Table 2). Even at day 6, HMW-NGF treated wounds differed significantly from other topically treated wounds of sialadenectomized animals ($p < 0.01$). By day 8 and beyond, NGF treated wound areas were comparable to those of sialadenectomized mice.

5.4. Discussion

Our findings that wounds heal faster in mice with intact submandibular glands, compared to sialadenectomized animals are in complete agreement with the results of Hutson et al. (1979); however Hutson and co-workers showed this in female mice, and we showed the same in male mice, which saliva contains much more NGF. Hutson et al. studied also the effect of duct ligation of the submandibular salivary glands on wound contraction, and they showed that in these animals the wound contraction was the same as in sialadenectomized animals. Our results and the results of Hutson et al., indicated that something in saliva influenced the wound healing in mice, and that the licking process is somehow involved in accelerating the rate of wound healing. We observed the animals licked each others wounds during the experiment. The experiments of Shen and co-workers (1979) demonstrated also that factors found in saliva seem to play an essential role in wound healing. Their data indicated that deprivation of saliva delays healing of gingiva wounds in rats. Since NGF has been isolated from granulation tissue (Levi-Montalcini et al., 1968) and possesses kallikrein-like action on smooth muscles

Table 1. EFFECTS OF SIALADENECTOMY AND CAGING SHAM-OPERATED AND SALIVARY GLAND EXCISION ANIMALS TOGETHER IN ONE CAGE (MIXED GROUP), UPON RATES OF WOUND CONTRACTION.

	Sham-operated animals n=25	Salivary gland excision animals n=25	Mixed group animals n=18
Operation day	100%	100%	100%
Day 2	62.7 ± 3.2	88.1 ± 4.2*	65.7 ± 2.3
Day 4	58.6 ± 2.3	82.7 ± 2.4*	56.4 ± 1.9
Day 6	37.3 ± 3.8	70.2 ± 4.0*	40.0 ± 1.7
Day 8	23.6 ± 1.7	41.9 ± 2.6*	25.0 ± 1.2
Day 10	16.6 ± 1.7	24.6 ± 2.8	18.5 ± 1.0
Day 12	11.7 ± 0.6	15.7 ± 1.9	12.6 ± 0.7

Values are percentage of initial wound area (means ± S.E.M.).
*Difference from sham-operated and mixed group animals significant at $p < 0.001$.

Table 2. EFFECTS OF TOPICALLY TREATMENT OF WOUNDS IN MICE WITH NGF, BUFFER, INACTIVATED-NGF, TRYPSIN, 2.5 S NGF OR UROKINASE UPON RATES OF WOUND CONTRACTION.

	HMW-NGF	Buffer	Inactivated NGF	Trypsin	2.5 S NGF	Urokinase
Day 0	100%	100%	100%	100%	100%	100%
Day 2	68.7±3.1	82.3±2.4*	80.4±3.1*	84.8±2.7*	83.4±4.1*	83.8±2.7*
Day 4	62.1±3.2	74.1±2.8**	80.5±3.0**	78.3±2.4**	79.8±3.7**	79.7±2.9**
Day 6	55.7±2.4	59.5±3.1	62.2±3.2**	66.1±2.2**	64.0±3.4**	66.4±3.0**
Day 8	36.5±3.0	42.3±2.6	34.6±1.7	48.3±2.1	40.9±3.1	46.8±3.3
Day 10	24.7±4.1	24.3±1.9	20.0±1.4	25.7±1.8	23.5±2.2	22.2±1.7
Day 12	13.4±0.9	17.1±1.0	16.1±0.8	16.1±1.0	15.7±1.8	17.8±1.2

Each group consisted of 25 animals. All mice had the salivary glands excised.
Values are percentages of initial wound area (means ± S.E.M.).
* Difference from HMW-NGF treated wounds significant at $p < 0.001$.
**Difference from HMW-NGF treated wounds significant at $p < 0.01$.

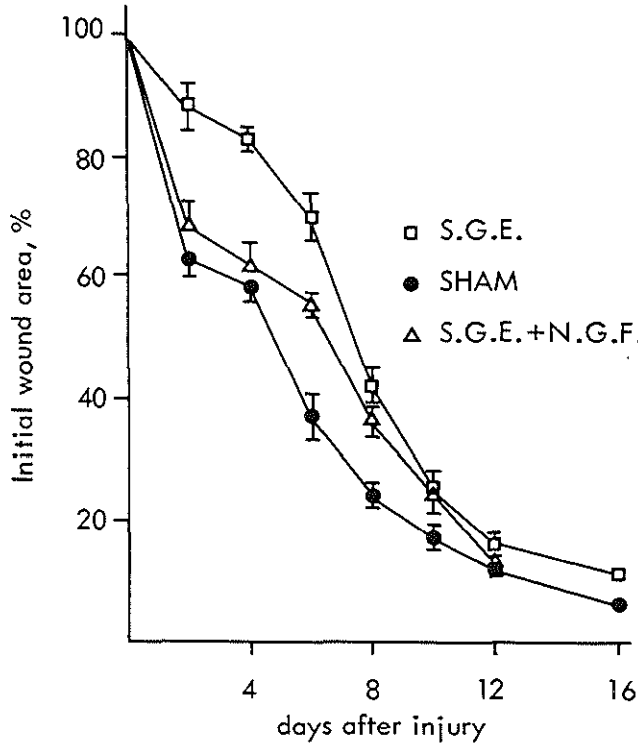

Figure 2.
Effects of sialadenectomy, sham-sialadenectomy and of NGF topically applied on wounds in sialadenctomized mice upon rates of wound contraction.
Values are means ± S.E.M.

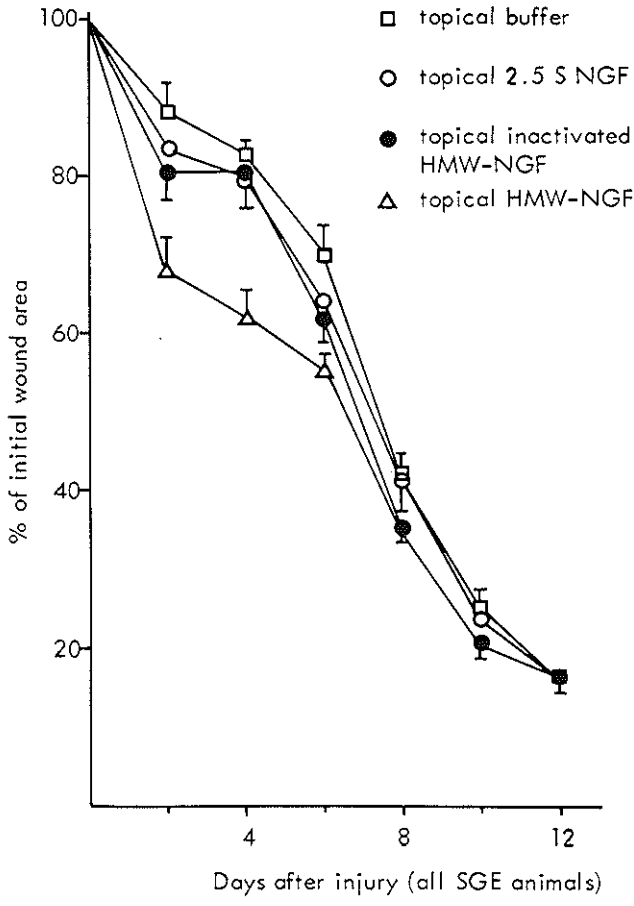

Figure 3.
Effects of buffer, 2.5 S NGF, inactivated HMW-NGF and HMW-NGF topically applied on wounds in sialadenectomized mice upon rates of wound contraction.
Values are means ± S.E.M.

(Bothwell et al., 1979), it perhaps stimulated contraction of myofibroblasts, which are believed to be responsible for wound contraction (Guber and Rudolph, 1978).

To evaluate the effect of HMW-NGF upon wound contraction, we applied a solution of this protein topically to wound areas of sialadenectomized animals immediately after injury, with three additional applications at 12 hours intervals. The concentration of HMW-NGF used was representative of that present in normal male mouse saliva (Murphy et al., 1977b). The results of our experiment indicate that NGF significantly accelerates the rate of wound contraction in animals without submandibular glands.

The enzymic active part of NGF, once activated by autocatalysis, can hydrolyse certain lysine and arginine esters. It also contains, as part of its subunit structure, the nerve growth promoting subunit 2.5 S NGF. To evaluate the role of the enzymic activity of HMW-NGF and of its nerve growth-promoting subunit upon rates of wound contraction, solutions of DFP-inactivated NGF and of 2.5 S NGF were applied topically to wounds in concentrations equimolar to those of the NGF found in saliva. Neither of these proteins displayed any significant effect upon the extent of wound contraction when compared to buffer-treated sialadenectomized animals. These findings suggest that the enzyme activity of the HMW-NGF molecule may be required for acceleration of wound contraction and that its nerve growth-promoting subunit is inactive in this experimental system. They further suggest that perhaps the plasminogen-activating property of HMW-NGF is responsible for the observed results. However, this seems unlikely since we have been unable to detect any effect of topically applied urokinase or trypsin (in concentrations equimolar to that of HMW-NGF) upon the rates of wound contraction in sialadenectomized mice. Urokinase and trypsin are respectively a well known plasminogen activating agent and an enzyme with esterase activity similar to HMW-NGF.

The combined observations that mouse granulation tissue contains NGF-like activity (Levi-Montalcini and Angeletti, 1968), that fibroblasts can secrete NGF (Young et al., 1975) and that NGF is present

in high concentrations in mouse saliva (Wallace and Partlow, 1976; Murphy et al., 1977a) suggest that secretion of NGF by cells prominent in granulation tissue may be important in the wound healing process. The results of this study indicate that promotion of wound healing may be one of the physiological functions of NGF in saliva.

CHAPTER 6

EXPERIMENT 4

DETERMINATION OF THE EFFECTS OF THE SUBMANDIBULAR GLANDS ON CHEMICALLY INDUCED COLON CANCER

6.1. Introduction

Among the several growth factors excreted in the submandibular saliva of the male mouse (Cohen and Savage, 1974; Bradshaw, 1978), Epidermal Growth Factor (EGF) and Nerve Growth Factor (NGF) have many influences in vivo and in vitro on all kinds of tissues (Levi-Montalcini and Angeletti, 1968; Cohen and Taylor, 1974).

In rats, mice and dogs, EGF, which appears to be identical to human urogastrone, can heal gastric stress ulcers (Koffman et al., 1977). EGF also increases the resistance of the gastric mucosal barrier to ethanol in rats (Pilot et al., 1979) and increases ornithine decarboxylase activity in the stomach, duodenum and colon in mice (Feldman et al., 1978). Ornithine decarboxylase is an enzyme, that plays an important role in the biosynthetic pathway of the polyamines - putrescine, spermidine and spermine. Polyamine production is an index of tissue growth since induction of these substances is closely related to the burst of intracellular activity preceding actual cell synthesis. Recently it is shown in adult male mice that EGF has a strong effect on DNA synthesis in the esophagus, stomach, small bowel and colon of mice (Scheving et al., 1979, 1980). DNA synthesis reflects cell proliferation since cells contain a fixed amount of DNA which is constant for any given species (Nundy et al., 1977).

NGF enhances neurite outgrowth of sympathetic ganglia in vivo and in vitro (Levi-Montalcini and Hamburger, 1953). High blood levels of NGF are found in patients with neurofibromatosis (Fabricant et al., 1979) and in children with neuroblastoma and fibrosarcoma (Burdman and Goldstein, 1964). Moreover, high blood levels of NGF are also found in patients with Paget's disease of bones while medical treatment of the disease showed marked decrease of these blood levels.

Recently considerable circumstantial evidence has been provided for the proposal that initiation of chemical carcinogenesis requires cell proliferation (Cayama et al., 1978). Previously we demonstrated that sialadenectomy decreased cell proliferation in small bowel (Li et al., 1980b). Since submandibular saliva of adult male mice contains high amounts of NGF and EGF (Murphy et al., 1979), both factors that influence cellular growth, we decided to study the effect of submandibular sialadenectomy on dimethylhydrazine-induced colonic cancer in mice.

6.2. Materials and methods

Swiss strain male mice, 40 days old, and weighing 26-28 g were fed and watered ad libitum, under alternating 12 hours cycles of light and dark for one week after arrival (n=200). They were then randomly assigned for submandibular sialadenectomy (n=100) or for a sham procedure. Operations were performed as described in Chapter 2.2. The animals were kept in cages with a closed bottom with sawdust bedding.

Three weeks after operation, a series of 20 weekly subcutaneous injections of 1,2-dimethylhydrazine (DMH) was started. The dose was 15 mg/kg in distilled water with final pH adjusted to 7.0 with 1 N NaOH. Slightly over half the mice were killed one week after the twentieth injection, the rest were killed ten weeks after the twentieth injection. After killing, the gut was removed from the ligament of Treitz to the anus, as described in Chapter 2.4. After flushing, the whole gut was inspected with the dissecting microscope and every area suspicious for tumor was removed and put in 10% formalin for histological examination. The whole body was examined for metastases. Histological sections were stained with haematoxylin and eosin. Specimens were coded to eliminate observers bias and examined by one pathologist.

6.3. Results

All but a few animals were in good health until the time of planned sacrifice. Shortly after the DMH-injections were started, 5 mice died, apparently from hepatotoxicity of the DMH as described by Haase et al.

(1973). Another 4 mice died prematurely following intussusception of large gut carcinomas. At operation the mean weights of the animals were not significantly different with a mean weight of 27.2 g in the sham group and of 27.1 g in the salivary gland excision group. At the start of the DMH treatment and on all time points later in the experiment there was a weight difference significant at $p < 0.001$ (Fig. 1).

At 20 weeks 71% of the sham-operated animals had neoplasms, almost exclusively in the distal half of the colon, vs. 47% of the sialadenectomized animals ($p < 0.02$ Fisher test) (Table 1). The number of tumors per tumor-bearing animal was 10% higher in the controls than in mice with submandibular gland excision ($p < 0.003$, t-test assuming Poisson distribution). Although the proportion of squamous and papillary tumors were the same, the number of tubular carcinomas was nearly halved by sialadenectomy ($p < 0.003$, t-test assuming Poisson distribution). The degree of differentiation of the malignant tumors was the same in both groups (Table 1). The areas of atypical hyperplasia were nearly halved in the sialadenectomized mice compared to the sham-operated animals.

At 30 weeks the number of mice with neoplasms was 95% in the sham-operated animals and 79% in the sialadenectomized mice ($p < 0.05$, Fisher test). The number of tumors per tumor-bearing mouse was 7.2 after sialadenectomy and 7.9 for controls (Table 1). Again the proportion of squamous and papillary carcinomas was nearly the same in both groups (Table 1). However, the number of tubular carcinomas was nearly 50% higher in the sham-operated group as compared to the ablation animals ($p < 0.001$, t-test assuming Poisson distribution). The degree of differentiation of the tubular and papillary carcinomas was nearly equal in both groups (Table 1).

Ordinarily, extra-colonic neoplasms following DMH-carcinogenesis in rodents occur in the ear canal. In these experiments there were none. Six of 8 extra-colonic neoplasms were vascular cancers of the liver, one was a vascular cancer of paratesticular tissue, and one was a signet cell carcinoma of the colon.

Table 1. NEOPLASMS AT SACRIFICE

	20 weeks		30 weeks	
	SGE	Sham	SGE	Sham
Mice with tumors	25	42	31	38
Mice without tumors	28	17	8	2
Total	53	59	39	40
Tumor/tumor-bearing mouse	2.16	2.42	7.16	7.92
Squamous carcinoma	2	6	19	16
Papillary carcinoma	6	8	15	10
Well diff.: no invasion	(3)	(2)	(8)	(4)
Well diff.: supererf.inv.	(2)	(4)	(0)	(0)
Well diff.: deep inv.	(0)	(0)	(0)	(0)
Moderate diff.: no invasion	(0)	(0)	(5)	(4)
Moderate diff.: superf.inv.	(1)	(2)	(2)	(2)
Moderate diff.: deep inv.	(0)	(0)	(0)	(0)
Tubular carcinoma	46	88	188	275
Well diff.: no invasion	(30)	(49)	(31)	(51)
Well diff.: superf.inv.	(8)	(10)	(0)	(3)
Well diff.: deep inv.	(0)	(0)	(0)	(0)
Moderate diff.: no invasion	(0)	(1)	(93)	(99)
Moderate diff.: superf.inv.	(8)	(27)	(58)	(108)
Moderate diff.: deep inv.	(0)	(1)	(0)	(5)
Poorly diff.: no invasion	(0)	(0)	(1)	(0)
Poorly diff.: superf.inv.	(0)	(0)	(0)	(6)
Poorly diff.: deep inv.	(0)	(0)	(5)	(3)
Total	54	102	222	301
Other carcinomas	0	0	4*	4**
Atypical hyperplasia	26	45	7	10
Metastases	0	1	2	0

* :all vascular cancers of liver.

**:two vascular cancers of liver, one vascular cancer of paratesticular tissue and one signet cell carcinoma.

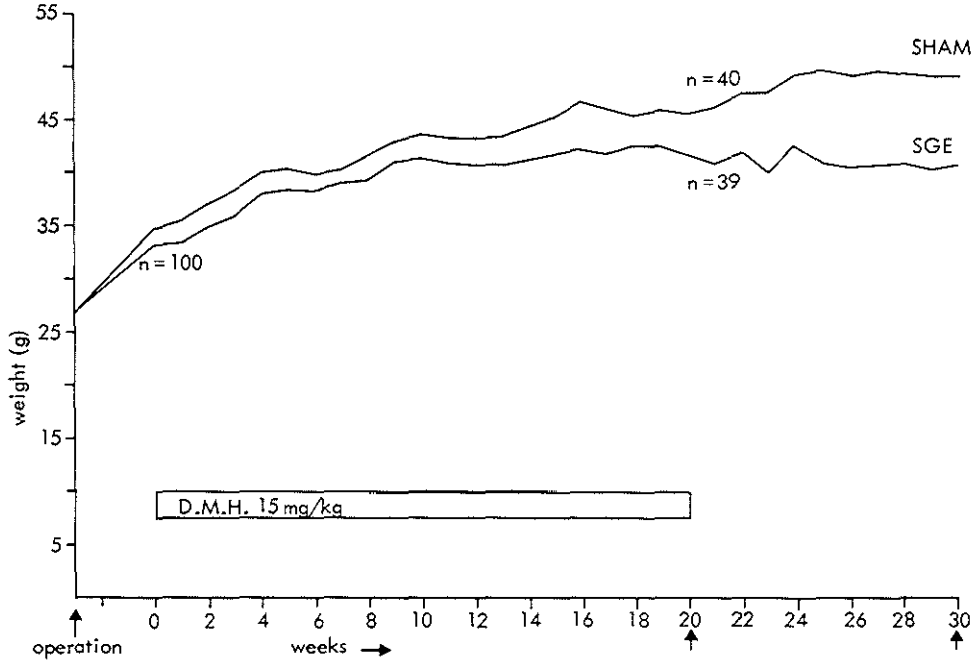

Figure 1. Curves of weekly weights of mice in chemically-induced colonic cancer study.
Each group started with 100 animals.
D.M.H. was administered weekly during 20 weeks.
Values are means.
Weight-differences are significant at $p < 0.001$ from start of D.M.H.-treatment till the end of the experiment.

6.4. Discussion

Removal of the submandibular glands from a male mice markedly inhibited the development of DMH-induced colonic neoplasia. Significantly more animals with tumors were found in the animals with intact submandibular salivary glands. The simplest explanation is that this response is a result of the suppression of colonic cell proliferation that follows submandibular sialadenectomy. In a previous experiment we demonstrated that sialadenectomy diminishes the rate of cell proliferation in mice by 13% in jejunum and 47% in ileum (Li et al., 1980b). Cayama and co-workers (1978) showed that there is strong evidence that chemically-induced carcinogenesis requires cell proliferation.

Stimulation of colonic cell division, by partial resection of the small bowel (Williamson et al., 1978; Oscarson et al., 1979), by distal diversion of pancreaticobiliary secretions (Chomchai et al., 1974; Williamson et al., 1978, 1979), or by local trauma (Pozharisski, 1975), all potentiate carcinogenesis. Inhibition of proliferation by starvation, or by weight loss associated with construction of a self-emptying blind loop inhibits carcinogenesis (Williamson et al., 1980). Nonetheless, the slower development of transplanted A-10 breast adenocarcinoma cells, or C-1300 neuroblastoma cells after submandibular sialadenectomy in male mice is not influenced by body weight nor nutritional status (Arnason et al., 1975).

Excision of the submandibular salivary glands in adult male mice will not remove the only source of EGF in the animal, but it abolishes the peak in plasma level seen after induced salivation (Byyni et al., 1974). Furthermore, excision of the submandibular salivary glands, which secrete EGF (Murphy et al., 1979) abolishes the main source of this growth promoting factor at the entrance of the gastrointestinal tract (Byyni et al., 1972).

Evidence points to a systemic metabolic depression following submandibular sialadenectomy in mice and rats; the slower gain in weight in sialadenectomized animals, as seen in this experiment, is not simply a matter of poor nutrition. The respiratory quotient of sham-operated

animals is significantly higher than that of sialadenectomized animals as described in Chapter 3. Even if sialadenectomized rats or mice are pair-fed, or fed by orogastric tube the same amount of food, difference in body weight persist, though the caloric content of feces is the same for both groups (Haldi and Wynn, 1963; Narasimhan and Ganla, 1968). Therefore we can expect that the food intake in both groups is the same (see also Chapter 3), and since we gave the DMH-dosage related to the body weight of each individual animal, the weight difference itself seems to have no effect on the tumor induction.

Thus the inhibiting effect of submandibular sialadenectomy may depend on absence of products of the submandibular gland either from the enteric lumen or from the systemic circulation. Recently Scheving et al. (1979, 1980) demonstrated that intraperitoneal administration of EGF stimulates cell proliferation in stomach, small bowel and colon in mice. Because all the described local effects of EGF on gastrointestinal mucosa, and on hepatic parenchymal cells are stimulatory, and because EGF is acid stable (Cohen and Taylor, 1974), EGF is an attractive candidate for a local regulator of enteric cell proliferation. An additional local component involved in carcinogenesis per se might be β-glucuronidase, which transforms conjugated metabolites of DMH into an active carcinogenic alkylating agent (Hawks and Magee, 1974; LaMont and O'Gorman, 1978). Changes in diet, of which saliva is a substantial constituent, do change the fecal content of β-glucuronidase. If the diets are high in fat or protein, the levels of bacterial β-glucuronidase activity in the large intestine increases in rats (Reddy et al., 1974, 1977; Bauer et al., 1979). Decreased flow of saliva after submandibular sialadenectomy might induce decreased local β-glucuronidase levels in the colon.

The development of malignant vascular neoplasms, independent of the presence of submandibular glands, is unusual in experiments involving parenteral administration of DMH, although benign hemangioendotheliomas of the liver are known after oral administration (Druckrey, 1970). The occurrence of malignant angiosarcomas in our animals may be due to ingestion of unaltered DMH or its metabolites from the urine (Fiala, 1975), because the animals were caged in cages with sawdust

bedding and coprophagia occurred.

Many epidemiological studies show that dietary factors are involved in the colonic carcinogenesis in man (Burkitt, 1971; Wynder and Reddy, 1974) and in experimental animal models (Freeman et al., 1978; Watanabe et al., 1978). It is possible that the proposed effects of diet are mediated by variation in saliva production.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

There is, as yet, no real explanation for the enormous rate of epithelial turnover of the mucosa of the digestive tract and how it is regulated to maintain a constant state in health. Intense and constant proliferation is found in production zones in the stomach, small intestine and colon, with migration of epithelial and other cells towards the lumen. In the rat, the mucosal renewal rate is 24 hours in the stomach, 48-72 hours in the jejunum and 24 hours in the ileum (Leblond and Walker, 1956). This proliferation appears to be in excess of that necessary to maintain surface integrity and may in some way be of functional importance.

The regulatory mechanisms of this proliferation are as yet not fully known. Humoral, neural and luminal factors have been studied (see Williamson, 1978 for review). Luminal factors seem to play a dominant part. Of these luminal factors, food, pancreaticobiliary secretions and gastric secretions have been studied most, and many of these factors seem to have an influence on intestinal mucosal hyperplasia (Altmann, 1974; Dowling, 1974; Williamson, 1978a,b).

However, compensatory growth still can occur without pancreaticobiliary secretions (Shellito et al., 1978) and without gastric secretions (Tilson et al., 1973; Tilson and Axtmayer, 1976). Small bowel, deprived of intraluminal food, and possibly intraluminal secretions, no longer maintains its normal morphology or function. Thiry-Vella fistula, a segment of small bowel that is excluded from the intestinal continuity without resection, demonstrates luminal narrowing and shows decreased cell proliferation of the mucosa (Gleeson et al., 1972). This atrophy can be completely reversed by restoring intestinal continuity. Starvation results in diminished epithelial proliferation in the bowel, even if the nutritional state is maintained by parenteral feeding (Levine et al., 1976). Starvation also diminishes the normal hyperplastic response after bowel resection in parenteral fed rats (Levine et al.,

1976). Intermittent starvation and periods of free access to food caused functional improvement, followed by small bowel mucosal hyperplasia in rats (Fabry and Kujalová, 1960); however animals thus fed gained less weight than normally fed rats.

If the intraluminal factors are not in pancreaticobiliary secretions or in gastric juice, they may be produced more proximal in the gastrointestinal tract. The fact that mammalian villi show a diminishing height from pylorus to ileo-cecal valve (Altmann and Leblond, 1970) suggests that a proximal factor is involved, produced in the stomach or even more orally. The hypothesis that an intraluminal trophic factor is secreted in the saliva, would be consistent with these observations. Starvation could presumably reduce, and hyperphagia could increase the amount of saliva secreted.

The biologically active protein, produced by certain mouse tumor cells, that stimulated rapid neurite outgrowth of sensory and sympathetic ganglia was isolated by Levi-Montalcini and Hamburger (1951, 1953), and subsequently named Nerve Growth Factor (NGF). This protein was shown to be identical to a protein purified from the submandibular salivary glands of mice (Levi-Montalcini and Angeletti, 1968; Oger et al., 1974). Nerve Growth Factor is also found in high concentrations in the saliva of the submandibular gland of the adult male mouse as well as in snake venoms (Levi-Montalcini and Angeletti, 1968). Extracts from other organs, including the parotid gland of adult male mouse had no such effect (Levi-Montalcini and Angeletti, 1968).

During the course of studies on Nerve Growth Factor from the submandibular salivary glands, Cohen, working in the laboratory of Levi-Montalcini, discovered yet another protein in the submandibular salivary gland, and later also in the submandibular saliva, responsible for accelerating incisor eruption and eyelid opening in new-born mice (Cohen, 1962).This factor when purified was called Epidermal Growth Factor (EGF) (Cohen, 1965).

Murphy et al. (1977a) determined that the submandibular gland of the male mouse was an exocrine rather than an endocrine organ for both these growth promoting factors NGF and EGF. Epidermal Growth Factor

like Nerve Growth Factor is only found in high concentrations in adult male mouse submandibular salivary glands and submandibular saliva and not in other salivary glands. This is the reason that in our studies attention was focussed on the submandibular glands.

Different investigators demonstrated that salivary gland excision inhibited systemic growth in different species of animals (Plagge, 1938; Bixler et al., 1955a,b). This weight difference was found even when the same amount of food was instilled by orogastric tube, while the caloric value of the fecal residue was found to be the same in animals with and without salivary glands (Haldi and Wynn, 1963). This growth inhibition could be reversed with Thyroid Stimulating Hormone (Wase and Feng, 1956a,b). Further evidence that the hormonal system is involved with the submandibular glands was the fact that the adrenal glands of sialadenectomized mice are heavier than those of control animals (Bixler et al., 1955b). Other workers found that sialadenectomized female mice were infertile (Hendry and Iversen, 1973). Narasimhan and Ganla (1968) concluded from an experiment in which duct ligation of the submandibular gland was also performed, that the influence of the gland was mainly endocrine. Since other investigators demonstrated that the glands had rather an exocrine than an endocrine function (Murphy et al., 1977b,1979) and showed that ablation of the glands had no influence on the plasma levels of NGF or EGF (Byyni et al., 1974; Murphy et al., 1977a), the study of the effects of the glands on systemic growth, metabolism and reproductive function presented in this thesis seemed relevant. The question whether this effect was mainly endocrine in nature or regulated by exocrine secretion in the saliva had to be answered.

In Chapter 3 the results of this experiment are described. Three groups of animals, all with the same mean weight at the time of operation, were insert in the study. The first group underwent a sham salivary gland excision, the second group a ligation of the duct of the gland, while in the third the submandibular glands were excised. The food intake in each group was studied during the first six weeks after operation, while all the animals had free access to food. We could

show that the intake was the same for each group of animals. After the third postoperative week, significant weight differences were found between the sham operated animals compared to the other two groups. The sialadenectomized animals and the duct ligated animals had nearly the same mean weight, and were significantly lighter than the sham-controls. This weight difference persisted till the fifteenth week postoperatively. These findings are in contrast with the results of Narasimhan and Ganla (1968), who found no weight differences between duct ligated and sham-operated mice. After the fifteenth week postoperatively, the sialadenectomized animals in our experiment remained lighter for the rest of the experiment, while the duct ligated group caught up in weight and after the 20th postoperative week there was no significant difference in weight between the duct ligated and sham-operated animals. One explanation for the growth spurt of the duct ligated group could be that some saliva still is produced by the gland and the saliva somehow reaches the mouth after the fifteenth postoperative week. Metabolically the duct ligated and sialadenectomized animals behaved in the same way 10, 20 and 30 weeks after operation. Their respiratory quotient differed significantly from the sham-controls. The respiratory quotients of the sham-operated animals indicated metabolism of a mixed diet, while the duct ligated and sialadenectomized animals were primarily utilizing fat. This finding is in agreement with Haldi and Wynn (1963) who showed that only less body fat was found in sialadenectomized animals with no other alterations in body constituents when compared to normal animals. In contrast to earlier experiments (Hendry and Iversen, 1973) we found that sialadenectomy did not influence the fertility in male or female mice. The conclusions of this experiment are that deprivation of saliva causes a derangement of metabolism, while fertility is not affected.

After we showed an effect of the submandibular glands on the metabolism of the animal and on the systemic growth, a study on the effect of the glands on specific growth was initiated. Since saliva reaches the stomach first in the gastrointestinal tract, the study of the influence of saliva on gastric stress ulceration seemed realistic.

Epidermal Growth Factor seems to be identical to urogastrone (Gregory, 1975). Urogastrone inhibits gastric acid secretion and EGF is demonstrated to heal gastric ulcers (Koffman et al., 1977). Both EGF and NGF are shown to stimulate ornithine decarboxylase activity, an enzyme essential for duplicating cells, in the stomach and duodenum of mice (Feldman et al., 1978; Greene and McGuire, 1978). Recently Scheving et al. (1979, 1980) demonstrated that EGF stimulates cell proliferation in the esophagus, stomach and small bowel of mice. Our own group found that saliva stimulated small bowel proliferation in mice (Li et al., 1980b). Although the exact mechanism of stress ulcers formation is not exactly known, gastric acid secretion seems to play an important role. Other factors that seem to be important are the cellular lining and mucous layer of the stomach (Butterfield, 1975). Therefore we expected that sialadenectomy would aggravate gastric stress ulceration, because of lack of saliva.

In the next experiment two groups of animals were used (Chapter 4). One group of sialadenectomized animals, the other group with a sham-operation performed. After a standardized stress procedure, an average of four stress ulcers was found in every stomach of animals either having a sialadenectomy or sham procedure performed previously. Since the restraint period, in which the ulcers seem to arise, was only 1.5 hours, the amount of saliva produced in this period could have been too small to show an effect. Therefore we stimulated the saliva production in both groups with isoproterenol, administered directly before restraint. Significantly more stress ulcers in the stomachs of sham-operated animals were found as compared to sialadenectomized animals. These results were totally unexpected, because all known effects of NGF and EGF were ulcer preventing. However, Otten et al. (1979) demonstrated recently that NGF stimulates the pituitary-adreno-cortical axis. Murphy et al. (1979) recently also showed, that animals with hippocampal lesions, which caused high corticosterone blood levels, combined with restraint, developed significantly more stress ulcers than control animals. Thus the explanation of the findings in our experiment could be that NGF stimulates the pituitary-adreno-cortical axis, and the high levels of

corticosterone combined with the restraint results in a greater number of gastric ulcers.

Another form of specific growth that can be studied, is wound healing. Since animals lick their wounds, an effect of saliva on wound healing cin be expected. The moistering, cleansing and antibacterial functions of saliva have been demonstrated (Van Kesteren et al., 1942; Bibby 1949). Hutson et al. (1979) indicated that wound contraction in female mice is retarded by sialadenectomy or duct ligation. They presumed that a factor or factors in saliva accelerated wound healing. Shen et al. (1979) suggested in their experiment in sialadenectomized rats that saliva had an effect on gingiva healing (healing by second intention), however no influence was shown on healing by first intention. Inflammation, granulation tissue formation, contraction and epithelialization, all play a part in normal wound healing (Edwards and Dunphy, 1958). Wound contraction is a major component in the closure of open skin wounds, and is believed to be caused by myofibroblasts (Guber and Rudolph, 1978). NGF possesses kallikrein-like actions and induces contraction in the isolated rat uterus (Bothwell et al., 1979). Therefore, it could be that NGF stimulates contraction of myofibroblasts in wounds. EGF may further stimulate epithelialization because it is thought to have a trophic effect on epithelial cells of skin (Angeletti et al., 1964).

The experiment described in Chapter 5 was performed to test these suggestions. The experiment of Hutson et al. was partly repeated. Open skin wounds of 1 cm^2 were made on the backs of mice in such a way that the animals were unable to lick their own wounds. Three groups of male mice were formed. One group of sialadenectomized animals, a second group of sham-operated animals and a third, mixed group of animals, of whom half were sialadenectomized and half sham-operated. Each group of animals was separately caged and wound contraction was followed by photographs. We observed that animals licked each others wounds. The wound contraction in the sialadenectomized group was significantly retarded compared to the other two groups, which showed the same degree of wound contraction. We concluded from this experiment that something in saliva accelerated wound contraction. Since we cooperated with the Laboratory of Physical Biochemistry at the Massachusetts General Hospital we were

able to use purified Nerve Growth Factor from submandibular glands. In sialadenectomized animals it was found that the enzymic part of NGF had the same influence on wound contraction as saliva, if administered topically every 12 hours during the first 36 hours after wounding. Purified EGF was unfortunately not available to test topically.

The last form of specific growth that we studied in relation to the submandibular glands, was the influence on chemically-induced colonic cancer. Sialadenectomy has been shown to decrease sponge-induced granulations in rats (Teixeira et al., 1976), and sialadenectomy decreased the growth of transplanted breast tumors in mice (Arnason et al., 1975). Excision of the submandibular salivary glands abolishes the main source of the growth promoting factors at the entrance of the gastrointestinal tract (Byyni et al., 1972).

Epidermal Growth Factor is known to enhance chemical carcinogenesis of the skin in mice (Reynolds et al., 1965; Rose et al., 1976). EGF heals gastric ulcers (Koffman et al., 1977), influences the gastric mucosal barrier (Pilot et al., 1979), and stimulates cell proliferation in stomach, small bowel and colon (Scheving et al., 1979, 1980). In contrast Nerve Growth Factor doubles the number of gastric stress ulcers per mouse (Chapter 4), although it accelerates wound healing in mice with submandibular sialadenectomy (Chapter 5).

In a previous study we demonstrated that sialadenectomy diminished the rate of cell proliferation in mice by 13% in jejunum and by 47% in ileum (Li et al., 1980b). Because susceptibility to probably all chemically-induced cancers varies directly with the rate of proliferation in susceptible cells (Cayama et al., 1978; Weinstein, 1979) the effect of removal of the submandibular glands on 1,2-dimethylhydrazine-induced colonic cancer was studied.

In Chapter 6 an experiment is described in which two groups of animals, the first sialadenectomized animals, the second sham-operated animals were compared. Both groups of animals were injected weekly with 1,2-dimethylhydrazine (15 mg/kg) for 20 weeks. Thereafter nearly half the animals in both groups were killed. The other animals were killed 10 weeks later. The whole alimentary tract was removed and examined

under the dissecting microscope. Every area which looked like a tumor was removed and histilogical specimens of this area were examined by a pathologist. The number of animals with tumors, both at 20 and 30 weeks, was significantly higher in the control group as compared to the sialadenectomized animals. The number of tubular carcinomas was also significantly higher in the sham-operated animals compared to the sialadenectomized group 20 and 30 weeks after starting of 1,2-dimethylhydrazine treatment. No significant differences in differentiation of the malignancies or number of metastases were observed between the groups.

The conclusion from this experiment seems to be that sialadenectomy inhibits chemical carcinogenesis in mouse colon. This effect seems to depend on absence of products of the submandibular gland. Since many studies showed that dietary factors are involved in colon cancer in man (Burkitt, 1971; Wynder and Reddy, 1974) and in animal models (Freeman et al., 1978; Watanabe et al., 1978), the change in amount of saliva produced could have this impact on colonic cancer.

In conclusion all 5 objectives formulated in the introduction to this thesis seem to be accomplished.

1. The submandibular glands have a stimulating effect on systemic growth of mice. The metabolism is changed after sialadenectomy or duct ligation of the gland. Sialadenectomized mice are utilizing more fat in metabolism than sham-operated controls. Sialadenectomy has no influence on fertility in mice.

2. Since ligation of the submandibular ducts has the same effect as sialadenectomy on systemic growth during the first 15 postoperative weeks, and on metabolism for a longer period, the conclusion that the saliva itself is more important in this regulation than humoral factors produced by the glands in mice seems valid.

3. Since stimulation of submandibular saliva production in sham-operated animals nearly doubles the number of gastric stress ulcers compared to stimulated sialadenectomized animals, it can be concluded that submandibular saliva aggravates gastric stress ulceration in mice.

4. Submandibular gland excision decreased wound contraction. Animals without these glands, but licked by animals with intact glands, show normal wound contraction, so that the conclusion has to be that saliva increases wound contraction in mice.

5. Since submandibular gland excision decreased the number of animals with chemically-induced colon tumors, as well as the number of tubular carcinomas in these animals, it seems to be that submandibular gland excision inhibits chemical carcinogenesis in the colon of mice.

SAMENVATTING

In het speeksel van de glandulae submandibularis van de manlijke muis komen twee groeifactoren, de Nerve Growth Factor (NGF) en de Epidermal Growth Factor (EGF) in zeer hoge concentraties voor. Verschillende studies hebben aangetoond dat deze groeifactoren afzonderlijk een invloed hebben op de proliferatie van diverse soorten cellen. Tot nog toe was echter weinig onderzoek verricht naar de invloed van speeksel, bijvoorbeeld door middel van sialadenectomie, in het dierexperimentele model. In dit proefschrift werd met name de invloed van sialadenectomie op cellulaire groei in de muis bestudeerd.

In Hoofdstuk 1 wordt een overzicht gegeven van de literatuur betreffende speekselklieren, terwijl tevens het effect van extirpatie van de glandulae submandibularis aan de hand van zover bekende literatuurgegevens wordt beschreven.

Hoofdstuk 2 getiteld "EXPERIMENTAL DESIGN AND METHODS" beschrijft de proefopzet, de proefdieren die gebruikt zijn in de eigen onderzoekingen, en tevens worden in dit hoofdstuk de chirurgische procedures die in de experimenten zijn toegepast beschreven. De gebruikte statistische bewerkingen worden vermeld, evenals de wijze van histologische onderzoek en de gebruikte chemische stoffen.

In Hoofdstuk 3 wordt het eerste eigen experiment beschreven. Dit onderzoek toont aan dat verwijdering van de beide glandulae submandibularis of onderbinding van de uitvoergang van de klieren, een achterblijven van de groei tot gevolg heeft vergeleken met controledieren. Tevens wordt in dit experiment aangetoond dat de stofwisseling van de muis wordt beïnvloed, terwijl daarentegen, in tegenstelling tot reeds bekende literatuur gevonden wordt dat de vruchtbaarheid door klierextirpatie niet wordt beïnvloed.

Hoofdstuk 4 beschrijft het tweede eigen experiment. In dit hoofdstuk wordt aangetoond dat speeksel geproduceerd door de glandula submandibularis en speciaal de Nerve Growth Factor daarin, het ontstaan en het aantal stress ulcera in de maag van de muis doet toenemen.

Hoofdstuk 5 geeft de resultaten weer van een onderzoek naar de invloed van speeksel op wondgenezing. Aangetoond wordt dat speeksel de wondgenezing bij muizen bevordert. In dit experiment wordt tevens aangetoond dat de gezuiverde Nerve Growth Factor lokaal op een wond toegediend de wondgenezing bij dieren zonder glandulae submandibularis bevordert, wanneer vergeleken met de wondgenezing bij controledieren.

Hoofdstuk 6 beschrijft het laatste eigen experiment. Bij muizen, waarbij door middel van 1,2-dimethylhydrazine toediening coloncarcinomen worden opgewekt, blijkt dat sialadenectomie het ontstaan van coloncarcinomen doet afnemen.

In Hoofdstuk 7 volgt tenslotte een algemene bespreking. Samenvattend kan geconcludeerd worden dat speeksel, zeker bij de muis een uitgesproken regulerende werking heeft op celproliferatie. Aan het eind van dit hoofdstuk worden de vragen die aanleiding gaven tot de in dit proefschrift beschreven onderzoek, en die in de "INTRODUCTION" zijn geformuleerd, beantwoord.

SUMMARY

In saliva of the submandibular glands of male mice high amounts of Nerve Growth Factor (NGF) and Epidermal Growth Factor (EGF) are found. These growth promoting factors are shown to influence the proliferation of different kinds of normal and malignant cells. Less attention has been given to the influence of sialadenectomy on cellular growth till now.

Chapter 1 reviews the literature of the salivary glands, and the effects of salivary gland excision in different kinds of animals.

Chapter 2 called "EXPERIMENTAL DESIGN AND METHODS" describes the experimental set-up, the animals used in the different experiments and the surgical operations performed in the different experiments. Furthermore in this Chapter the statistical analysis used, the histological analysis performed and the biochemicals used are presented.

Chapter 3 describes the first experiment. In this experiment it is demonstrated that salivary gland excision or duct ligation inhibits systemic growth of the animals. Furthermore it is shown that there is a change in metabolism after salivary gland excision, while fertility is not influenced.

The second experiment is described in Chapter 4. The number of gastric stress ulcers after salivary gland excision is diminished, while Nerve Growth Factor, equimolar to that produced in saliva during stress, instilled in the stomachs of sialadenectomized mice,is shown to aggravate stress ulceration.

In Chapter 5 the data are given of an experiment in which the influence of saliva on wound healing was studied. It is shown that saliva stimulates wound contraction, the same happened in sialadenectomized animals if NGF was topically applied on the wounds.

Chapter 6 described the fourth experiment. It could be demonstrated that sialadenectomy inhibited the occurrence of chemically induced colon cancers.

The last Chapter, named "GENERAL DISCUSSION AND CONCLUSIONS" describes the results of the own experimental work in relation to the relevant data from the literature. The conclusion seems to be that saliva at least in mice has a regulatory effect on cellular growth. This chapter ends with an answer to the questions which initiated the studies presented in this thesis and which were formulated in the INTRODUCTION.

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