STUDIES OF TUMOR PROMOTERS AND DRUG-METABOLIC ENZYMES IN HAMSTER BUCCAL POUCH MUCOSA

by

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B.D.Sc., Beijing Medical College, 1983

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCES

in

THE FACULTY OF GRADUATE STUDIES
(OMSS, Faculty of Dentistry)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

February 1993

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Date 29 April 1993

DE-6 (2/88)

Abstract

Lack of tumor promoters has been the major obstacle in the use of the hamster buccal pouch mucosa (HBPM) model. Two experiments were designed to investigate the effects of two new mouse skin tumor promoters, okadaic acid (OA) and methyl methanesulfonate (MMS), on HBPM.

Short term effects of OA were studied. A single application of 10 μg of OA in 0.1 ml of acetone produced marked inflammation as well as an increased mitotic rate ($p<0.01$) as compared to that of the control. It, therefore, seems that OA possesses some essential properties of tumor promoter. Long term study is necessary to prove that it is a potent tumor promoter in HBPM.

Tumor promoting effects of MMS were examined in a long term experiment. 25 hamsters were divided into 3 groups. In group I, pouches of 10 hamsters were initiated with 7,12-dimethylbenzanthracene (DMBA), then promoted with MMS for 10 weeks. In group II, pouches of 10 hamsters were initiated with MMS and promoted with MMS. In group III, pouches of 5 hamsters were initiated with DMBA (right pouches) or MMS (left pouches), and promoted with acetone. The results showed that MMS had moderate tumor promoting effects but no tumor initiating effects in the model.

Gamma-glutamyl transpeptidase (GGT) and placental glutathione S-transferase (GST-P) have been found to be increased during HBPM carcinogenesis. Whether such increases are oncofetal remains to be answered. There are few studies on the normal distribution of GGT and GST-P in hamster tissues. One experiment

was designed to study their tissue distributions during the development of hamster pouches and several selected organs and tissues. The results showed no GGT and GST-P activities in hamster pouches during their development. The expression of GGT and GST-P activities during HBPM carcinogenesis may represent an acquired genetic alteration instead of oncofetal reversion. GGT was found in epithelial cells, particularly those with 'brush borders', in several organs and tissues, supporting the hypothesis that GGT may participate in amino acid transportation. Rarely, GGT was also noted in mensenchymal cells. GST-P was observed in few organs and only expressed in epithelium.

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LITERATURE REVIEW

I. Chemical carcinogenesis

1. Multistage carcinogenesis

Sir Percival Pott (1775) first noted a connection between human skin cancer and exposure to soot. Years later, Yamagiwa and Ichikawa (1918) succeeded in introducing tumors in rabbit ears by repetitive topical application of crude coal tar. Since then, several groups of chemical carcinogens have been identified and a number of experimental animal models for the study of carcinogenesis have been established. Among the carcinogens, polycyclic aromatic hydrocarbone (PAH) carcinogens is the most common environmental carcinogens causing human cancers including oral cancers (Dipple, 1985). Cramer and Stowell (1943) showed that a single large application of carcinogen 20-methylcholanthrene, a kind of PAH, induced tumors in mouse skin. It was found that, in the mouse skin model, a single subthreshold dose of a carcinogen followed by repetitive application of noncarcinogenic irritants, such as croton oil and wounding, also produced tumors (Berenblum and Shubik, 1947a,b). It is now generally accepted that carcinogenesis is a multistage process in several animal systems, consisting of the distinct and sequential stages of initiation, promotion, and progression (Friedwald & Rous, 1944; Berenblum & Shubik, 1947a,b; Marks & Fürstenberger, 1987).

Initiation

Initiation refers to a permanent DNA change in one or a few cells that have been exposed to a carcinogen at a level that is insufficient to cause a neoplasm (Boutwell, 1989). This permanent change is generally considered to be caused at a genetic level. Brookes and Lawley (1964) confirmed the somatic mutation theory by demonstrating that PAH carcinogens bind covalently to cutaneous DNA, and that the binding capacity of the carcinogen correlated with its tumorigenicity. This theory has been supported by many other studies (Bishop, 1982; Weinberg, 1982; Bister & Jansen, 1986; Sell et al., 1987; Yuspa & Poirier, 1988). DNA synthesis at this stage is probably required for fixation of the mutant gene in daughter cells and thus is responsible for the irreversibility of the initiated cells (Cayama et al., 1978).

Promotion

Promotion has been described as the reversible clonal expansion of previously initiated cells that grow faster than the surrounding normal cells so as to develop into a visible neoplasm (Boutwell, 1989; Pitot et al., 1989). In contrast to initiation, tumor promotion appears to proceed along an epigenetic route, which could be evoked by either a promoter or a complete carcinogen. The latter has both tumor initiating effects and tumor promoting effects (Quintanilla et al., 1986; Brown et al., 1986). However, there is evidence indicating that DNA damage or mutation also occurs during the promotion process. Contrary to initiation, promotion occurs over a long period of time, is reversible at early stages (Boutwell, 1964),

and shows a distinct threshold below which promotion effects will not be observed (Diamond et al., 1980). The prolonged process, the presence of a threshold and early reversible nature of promotion make it the most important step in the study of carcinogenesis, as interruption of tumor promotion is much more feasible clinically as compared to that of the rapid, irreversible process of tumor initiation (Slaga et al., 1980).

Boutwell (1964) has further divided promotion into two stages. The first of which is conversion, whereby initiated cells are converted to the dormant tumor cells. This stage is promoter-specific because it can be induced only by either a complete carcinogen or a noncarcinogenic promoter. The second stage is propagation, whereby the dormant tumor cells multiply to form a tumor mass. This stage is less specific. Not only complete carcinogens and tumor promoters but also hyperplasiogenic or mitogenic agents may turn the dormant tumor cells into a tumor. This subdivision of promotion has been supported by a number of subsequent studies (Slaga et al, 1980; Fürstenberger et al., 1981).

Although tumor promoters have no tumorigenicity when tested alone, they remarkably increase tumor yield when repetitively used following initiation. Tumor promoters exhibit a broad spectrum of biological effects (Johnson et al., 1987; Marks et al., 1988; Marks, 1990). The essence of promotion is, however, believed by many investigators to be the selective stimulative growth force for the initiated cells relative to the surrounding cells (Solt & Farber, 1976; Farber, 1984). In skin, all tumor promoters induce hyperplastic changes, supporting the hypothesis that epidermal hyperplasia is

essential for promotion. However, not all skin hyperplasiogenic or hyperproliferative agents are promoters.

The extensive studies of phorbol esters, particularly 12-0-tetradecanoylphorbol-13-acetate (TPA), have yielded some results regarding the biochemical events and mechanisms of tumor promotion. In mouse skin, two prominent events occur almost immediately after treatment with phorbol esters: one is the induction of the arachidonic acid cascade, resulting in production of prostaglandins and metabolites along the lipoxygenase pathway, and another is the activation of protein kinase C (PKC). Prostaglandins are involved in the induction of epidermal hyperproliferation. The activation of PKC by the substitution of TPA promoter for diacylglycerol opens the signal transduction pathway normally controlled via growth factors (Johnson et al., 1987) and results in epidermal hyperplasia (Marks & Fürstenberger, 1987). Other umor promoters that are structurally dissimilar to phorbol esters have also been found to act through the PKC pathway and therefore are called TPA-type of tumor promoters. Tumor promoters that act through other mechanisms are called non-TPA-type tumor promoters, which may affect phosphoprotein phosphotase, such as okadaic acid, leading to amplification of protein phosphorylation (Haystead et al., 1989), or may induce a long-lasting increment of intracellular Ca^{2++}, such as detergent (Setälä et al., 1954), organic peroxide (Kensler & Taffee, 1986) and thapsigargin (Thastrup et al., 1990), evoking the skin wound response.

Progression

The stage of progression is believed to be characterized primarily by its karyotypic instability and evolution to malignancy. The development of irreversible, aneuploid malignant neoplasms distinguishes progression from both initiation and promotion. This process is generally considered to be the effect of the accumulative gene mutations, as it may be augmented by treating papilloma with initiators (Hennings et al., 1983).

2. Tumor marker

Tumor markers have been defined as the specific biochemical and/or molecular characteristics, products, and changes produced in a host suffering neoplasia (Beer & Pitot, 1987). Neoplasia also could be characterized by a loss of normal cellular markers (Miller & Miller, 1974).

The significance of studying tumor markers lies in three aspects: (1) Since the recognizable morphological changes for potential malignant transformation occur late in the multistep process, it is essential to develop markers such as enzymes to label the carcinogen-altered cells from the early stages of carcinogenesis in order to study them. (2) Since only a small percentage of the premalignant lesions will develop into malignancy, it is very important to develop tumor markers for an evaluation of the malignant transformation potential of premalignant lesions, and for the early diagnosis of cancer. (3) An understanding of the key enzymatic changes may lead to a natural approach to intervene the

process at the early stage of carcinogenesis through specific chemotherapy and thus to alters the enzymatic activity.

Tumor markers have been studied most extensively in the rat liver model and multiple tumor markers have been identified. The preneoplastic and neoplastic cells are characterized by their phenotypic diversity. The availability of multiple tumor markers may lead to the detection of those phenotypes which have the greatest propensity to progress to carcinomas and of those treatments which increase such lesions. The availability of multiple tumor markers is also important in screening for different carcinogens, promoters or progressors since different classes of these agents may promote different initiated cells which express certain markers. In the liver, it has been reported that altered hepatic foci (AHF) expressing multiple markers may have a faster growth rate and are more autonomous than single marked AHF.

Malignant tumors are known to share many features with normal embryonic tissue, such as possession of enzymes and proteins that are normally present in embryonic tissues but low or absent in normal adult tissues. These enzymes or proteins are called carcino-embryonic (oncofetal) proteins or markers. Generally, the amount of oncofetal proteins present in neoplastic cells is higher than their normal embryonic counterparts.

The oncofetal hypothesis of carcinogenesis is derived from findings that there are analogies between the differentiation of normal fetal tissues and the reverse, the loss of differentiation by tumors (Knox, 1972). It has been proposed that the appearance of oncofetal protein may reflect de-differentiation of neoplasia

(Novogrodsky et al., 1976). The hypothesis, therefore, suggests that cancer may be viewed as a problem in normal biological development. Studies of the relationship between cancers and their normal embryonic tissues may lead to a better understanding of the mechanisms of malignant transformation and the regulation of embryogenesis.

3. The HBPM model

Since Salley's discovery of the HBPM model in 1954, this system has remained the most useful one in the study of oral carcinogenesis. The cheek pouches are bilateral evaginations of oral mucosa and provide ample mucosal tissue. Contralateral pouchs can serve as controls and the pouches are easily visualized and accessible. The pouch epithelium is susceptible to a number of carcinogens, including 7,12-dimethylbenzanthracene (DMBA) which produces predictable stepwise changes in the mucosa and a consistent production of carcinomas in all treated animals (Salley, 1954; Morris, 1961). A number of studies have shown that there is a consistent time frame of tumor development with triweekly painting with 0.5% DMBA. Dysplasia develops around 6 to 8 weeks, early squamous cell carcinomas (SCCs) develop around 8 to 10 weeks and invasive SCCs develop around 10 to 12 weeks (Salley, 1954; Morris, 1961; Shklar, 1972; Freedman & Shklar, 1978). It has been shown that the sequential changes of hyperkeratosis, dysplasia and squamous cell carcinomas in the HBPM during carcinogenesis are comparable to human oral lesions of epithelial hyperplasia, dysplasia and carcinoma (Santis et al., 1964; MacDonald, 1973).

Another advantage of using hamsters in the study of carcinogenesis is that the incidence of spontaneous neoplasms in hamsters is much lower than those in mice and rats (Van Hoosier & Trentin, 1979; Pour et al., 1979), with only rare cases having been reported in the literature (Zhang, 1989), and none of these were squamous cell carcinomas.

DMBA carcinogenesis in HBPM is also susceptible to a number of cocarcinogenic agents, particularly to those presumptively associated with human oral carcinogenesis, such as alcohol and chronic mechanical irritation. There is, however, no potent promoter found for this model, although weak promoting agents such as benzoyl peroxide have been identified. The prototype mouse skin promoters, such as TPA, are ineffective in the HBPM model. An in vitro study by O'Brien and Diamond (1979) showed that TPA did not affect cell growth or DNA synthesis of hamster cells as it did in mouse epidermal cells. Additionally, hamster cells rapidly inactivated TPA, while there was little, if any, such metabolism in mouse skin epidermis. This may explain why TPA is an ineffective promoter in the HBPM carcinogenesis. As discussed above, promotion is a very important step in carcinogenesis and is critical in determining if a tumor will develop. The lack of an effective promoter has been the biggest obstacle in exploring many fundamental aspects of carcinogenesis using the HBPM model.

4. New tumor promoters

The phorbol ester group of tumor promoters has been extensively investigated and the results of these studies have

contributed tremendously to our understanding of tumor promotion. In the past several years, there has also been considerable interest in non-TPA type tumor promoters in the mouse skin model. These studies of new promoters have greatly improved our understanding of tumor promotion and have provided possibilities that some of them are also potent tumor promoters for HBPM model.

Okadaic acid

Okadaic acid (OA $C_{44}H_{68}O_{13}$) is a polyether fatty acid with a m. wt. of 805.02. It has been first isolated from two marine sponges (Tachibana et al., 1981) and later found in several types of marine plankton, which are the food of these marine sponges (Murakami et al., 1981; Yasumoto et al., 1984). It causes skin irritation and gastroenteritis in humans (Murata et al., 1982). Recently, it han been shown to be an effective tumor promoter in the mouse skin model (Suganuma at al., 1988), and in some cell culture systems (Redpath & Proud, 1989).

While phorbol ester group of tumor promoters such as TPA, and other TPA-type of tumor promoters are believed to function mainly through binding with and subsequent activation of PKC, OA does not bind with PKC. Studies have shown that OA is a very potent inhibitor of serine/threonine-specific protein phosphatases 1 and 2A in any cellular event (Cohen, 1989), but has no direct inhibiting effect on the activity of any of eight known protein kinases. Since these two protein phosphatases are the chief enzymes that reverse the action of PKC, their inhibition causes a net increase of phosphorylated proteins (Haystead at al., 1989).

Methyl methanesulphonate

Methyl methanesulphonate (MMS $C_2H_6O_3S$) is an alkylating agent (AA) with a m. wt. of 110.13 and covalenty binds to the chemical groups of biological molecules that have an excess of electrons (nucleophiles). This binding is known as alkylation (Kohn, 1979). MMS is a direct-acting compound and does not require metabolic activation (Kleihues & Coopers, 1976; Garte et al., 1985). It has been found that MMS shows carcinogenic effect in a number of animal models, such as the rat nasal mucosa model. This effect is usually observed after a long term treatment (IARC, 1974; Sellakumar et al., 1987). In contrast, in some other models, MMS has been shown not to exhibit any tumor initiating effects (Frei & Venitt, 1975; Pegg, 1983; Fürstenberger et al., 1989).

The reactivity of AAs toward nucleophiles can be defined in terms of reaction mechanisms and the dependence of reaction rates on the nucleophilic strength of the receptor atoms (Vogel & Natarajan, 1981). The reactivity of AAs has been expressed by the Swain-Scott substrate constant s, which is a measure of the sensitivity of AA to the strength n of the nucleophile with which it reacts. Studies have shown that there is a general, direct correlation between chromosome breaking efficiency, cytotoxicity and s value, and a general inverse correlation between s value and the ability of AAs to induce point mutation.

Studies have shown that mutagenicity of an alkylating agent correlates with its carcinogenicity (Newbold et al., 1980). The inefficiency of MMS as a tumor initiating agents in a number of models has been explained to be the result of its low mutagenicity

(Loveless, 1969; Newbold et al., 1980; Morris et al., 1982; Pegg, 1983; Natarajan et al., 1984;). MMS has a high *s* value, hence it has as same low mutagenic but high clastogenic and cytotoxic effects as do other AAs with high *s*. Low level production of O^6-guanine methylation by AAs with high *s* value, such as MMS, contributes to the low mutagenicity. An increased level of O^6-guanine methylation has been found to parallel increased mutagenicity (Frei & Lawley, 1976; Suter et al., 1980; Newbold et al., 1980). Also, it has been proposed that the low mutagenicity of AAs with high *s* is because (1) they inhibit SH-group and, (2) they are very cytotoxic, causing death of the mutant cells (Vogel & Natarajan, 1981).

Although induction of mutations in somatic cells has been considered the most likely mechanism by which AAs might initiate neoplastic growth, other mechanisms can not be ruled out (Pegg, 1983). The possibilities include induction of latent viral genes by AAs, synergistic effects of AAs and viruses and alteration of host immunocompetence (Pegg, 1983).

In the mouse skin model, MMS is a rather powerful stage I (conversion) tumor promoter, although it is not carcinogenic by itself (Fürstenberger et al., 1989). This is not surprising since clastogenic effects are characteristic of stage I promoters. The correlation between clastogenicity and conversion in tumor promotion has been reviewed in terms of induction of prooxidant states (Cerutti, 1985), which is critical in the generation of chromosomal aberrations in skin tumor promotion (Füsternberger et al., 1989).

II. Gamma-glutamyl transpeptidase (GGT)

I. Structure

Gamma-glutamyl transpeptidase (GGT) is a plasma membrane bound glycoprotein elaborated in endoplasmic reticulum and transported to the plasma membrane via the Golgi apparatus (Ishii et al., 1986). It is composed of two subunits which are located on the extracellular side of cell membrane (Horiuchi et al., 1978; Marathe et al., 1979). The heavy subunit anchors GGT in plasma membrane (Matsuda et al., 1983), while the light subunit noncovalently binds to the heavy subunit (Hughey & Curthoys, 1976). Both of subunits are responsible for its catalytic function (Gardell & Tate, 1981).

2. Metabolic roles

GGT catalyzes the initial step in the utilization of glutathione (γ-glutamylcysteinylglycine, GSH) in which the γ-glutamyl moiety of this tripeptide is transferred to an acceptor, which may be an amino acid, dipeptide, or GSH itself (Tate & Meister, 1981). GGT is the major enzyme in γ-glutamyl cycle, a metabolic pathway that accounts for the enzymatic synthesis and degradation of glutathione (Elce & Broxmeyer, 1976; Meister, 1976; Samuels, 1977; McIntyre & Curthoys, 1979).

The biological significance of GGT is not entirely clear. Besides its role in GSH metabolism and the maintenance of intracellular GSH levels, participation in amino acid transport across cell membranes, detoxification of electrophiles, peptide storage, storage and transport

of cysteine, and cell proliferation have been suggested (Rosalki, 1975).

Gamma-glutamyl cycle as a possible amino acid transport system was first proposed by Orlowski and Meister in 1970. This theory was later supported by a large number of studies including studies on the location of GGT enzymes. Marked GGT activity has been found in the brush border of epithelial cells lining the proximal convoluted tubules and loops of Henle, in the surface epithelial cells of the small intestine, especially the jejunum, and in the choroid plexus. All of the epithelial cells in these locations function in active amino acid absorption, supporting the hypothesis that GGT may play an important role in amino acid transportation. Conflicting reports regarding GGT's role in amino acid transportation, however, also exist (Curthoys & Hughey, 1979).

A large number of studies has shown that GGT may have an important function in the detoxification of electrophiles through its role in maintaining intracellular GSH level and through the mercapturic acid pathway. The first step of the mercapturic acid pathway is the glutathione conjugation reaction, in which a group of isoenzymes known as glutathione transferases (GSTs) catalyzes the conjugation of electrophilic compounds with reduced glutathione, thus protecting macromolecules such as DNA from attack by carcinogenic agents (Degen & Neumann, 1978; Chasseaud, 1979; Moldeus & Jernstrom, 1983). The second step of this pathway is catalyzed by GGT which removes the γ-glutamyl moiety from the GSH conjugates to form cysteine derivatives, which subsequently undergo acetylation to form N-acetyl-L-cysteine derivatives (mercapturic

acids) (Curthoys & Hughey, 1979). The mercapturic acids are soluble and readily excreted through kidney and bile ducts (Boyland & Chasseaud, 1969), making this pathway to be one of the most important detoxification processes in the body.

3. GGT and carcinogenesis

GGT as a tumor marker has been studied extensively in liver and several other experimental models. It is significantly elevated in both preneoplastic and neoplastic hepatic lesions and in a variety of experimentally induced or in human premalignant and malignant epithelial lesions, such as human oral precancerous lesions and SCCs (De Young et al., 1978; Buxman et al., 1979; Fiala, 1979; Fiala et al., 1979a,b; Gerber & Thung, 1980; Uchida et al., 1981; Calderon-Solt & Solt 1985; Mock et al., 1987). However, human lymphocytic leukemia shows decreased GGT activity (Novogrodsky et al., 1976; Hultberg & Sjogren, 1980).

Of the biochemical markers for recognition of early preneoplastic lesions, GGT is one of the best studied. Elevated GGT activity has been noticed as early as after a single application of subcarcinogenic dose of hepatocarcinogens (Scherer et al., 1972; Scherer & Emmelot, 1975a,b, 1976; Hanigan & Pitot, 1985). GGT(+) cells may develope chromosome abnormality (Miyazaki et al., 1985). Moreover, the transcription of several proto-oncogenes during hepatocarcinogenesis has been studied. The expression of H-ras and c-myc gene has been found to be elevated in GGT(+) cells (Sinha et al., 1986).

The usefulness of GGT as a tumor marker has been explored in the HBPM model (Solt, 1981; Solt & Shklar 1982; Zhang & Mock 1987, 1989, 1992; Zhang, 1989). Normally the adult cheek mucosa epithelium is devoid of detectable GGT activity. However, hamsters treated with carcinogens progressively developed discrete foci of GGT positive cells. GGT activity disappeared with formation of overt neoplasms (Zhang & Mock, 1987; Zhang 1989).

The loss of GGT activity during multistep carcinogenesis has also been noted in liver, although it was much less dramatic and obvious (Tatematsu et al., 1988b). The loss of GGT staining in tumors may indicate a further step toward malignancy.

The exact role of GGT in carcinogenesis is not clear. It has been suggested that cells with higher GGT levels may have a better capacity of detoxification than those with lower GGT, resulting in a selective growth advantage required for further transformation (Laishes et al., 1978).

4. Tissue distribution

In most mammals, such as humans, laboratory rodents, brown bears, dogs, oxes, when adult tissues are assayed for GGT activity, the strongest GGT activity is noted in kidney, with weaker levels in pancreas, and still weaker activity in liver and negligible in other organs (Glenner et al., 1961). GGT activity in the same organ varies in different species of animals. After the introduction of histochemical methods for the localization of GGT, a number of studies have been performed to establish the localization of GGT in a variety of tissues and cells. The precise localization of transpeptidase is important in

view of its proposed roles in transport and detoxification processes, as well as its usefulness as an oncofetal marker (Tate & Meister, 1981). As summarized by Tate and Meister (1981), histochemical studies have shown that, in general, high GGT activity is seen in cells which exhibit secretory or absorptive functions, including the epithelial cells of renal proximal tubules, jejunum, duodenum, bile duct, epididymis, prostate, testis, seminal vesicles, choroid plexus, ciliary body, retinal pigment epithelium, bronchioles, thyroid follicles, mammary glands, hepatocytes of canalicular regions of liver, pancreatic acinar and ductile epithelial cells, post-secretory ameloblasts and odontoblasts of developing teeth, and the epithelium of the uterine endometrium (Marathe et al., 1979; Albert et al., 1961, 1964, 1966, 1970; Ruthenburg et al., 1969; Fiala et al., 1976, 1977; Adjarov et al., 1979; Dawson et al., 1979; Åhlund-Lindqvist & Lindskog, 1985). High GGT activity has also been noted in non-secretory or non-absorptive epithelial cells, such as the granular cells of the maturing ovarian follicle and the follicular sheath of growing hair (Buxman et al., 1979).

GGT activity in different organs and tissues also varies depending upon the stage of the development of an organ and tissue. In many species studied, including human, mouse, rat, rabbit, hamster, and guinea pig, fetal tissues in general exhibit much higher GGT activity than adult tissues with the exception of kidney, which is the main source of GGT in the adult (Albert et al., 1964, 1970).

GGT activity during the development of organs and tissues has been studied in detail in the rat and humans. Their fetal and neonatal brain, lung, and particularly liver, show much higher GGT

activity than that in the adult organs, whereas, there is a steady increase in the GGT activity in kidney during its development. Adult human kidney contains 11 times greater GGT activity than the fetal renal tissue (Albert et al., 1970a,b). Interestingly, studies have shown that hepatic and lung carcinomas developnig in rat, human and other mammals showed a marked increase in GGT activity (Tatematsu et al., 1985; Yamamoto et al., 1988), suggesting GGT enzyme activity in these organs is oncofetal in nature. On the contrary, renal cell carcinomas showed decreased GGT activity (Albert, 1965; Flemming et al., 1977; Tsuda et al., 1985),

In other organs and tissues, increased GGT in preneoplastic and neoplastic lesions has not been found to be oncofetal in nature (De Young et. al., 1978; Adjarow et al., 1979; Traynor et al., 1988).

Only one study has investigated GGT activity in adult hamster organs and tissues (Albert et al., 1964). The results showed that hamster kidneys contained the highest level of GGT and the pancreas showed a moderate amount of GGT. Only trace amounts, or no GGT were found biochemically in other organs and tissues, including liver, spleen, gastrointestinal tract, adrenals, ovary, uterus, epididymis, testicle, submandibular salivary gland, and lung. When examined histochemically, GGT activity was found mainly in the cells of the proximal convoluted tubules in kidney, in the external secretory portion of the cell cytoplasm of the secretory follicle in pancreas, and in the cylindric cells of the mucous membranes of the small and large intestine. Slight GGT activity was demonstrated in the cytoplasm of the glandular cells and of the secretory ducts. Although no GGT activity was noted in hepatocytes, a slight positive reaction was

occasionally observed in the wall of bile canaliculi and some reticuloendothelial cells. GGT activity was not observed histochemically in other organs, including stomach and lung. The results of this study have not been confirmed by other investigates and the study did not investigated GGT activity in hamster oral or nasal mucosa and skin. Several studies on oral carcinogenesis have shown that adult hamster pouches contain no demonstrable GGT activity. No study has investigated GGT activity in fetal or neonatal hamster tissues and organs, including hamster pouches, salivary glands, and odontogenic tissues.

III. Placental glutathione transferase (GST-P)

Glutathione S-transferases (GSTs) are a family of multifunctional proteins composed of dimeric subunits. They were found initially in rat liver (Booth et al., 1961) and later isolated from rat liver cytosolic supernatant fraction (Habig et al., 1974a), disclosing the multiple isoenzymes acting on a broad spectrum of universal substrates. Of many forms of GSTs identified in various organs of various species, rat, mouse and human's are best studied (Mannervik, 1985; Mannervik et al., 1985, 1987; Hayes et al., 1987).

GSTs had been named alphabetically in relation to isoelectric points and molecular weight (MW) of the subunits (Habig et al., 1974b; Bass et al., 1977). Later, they were divided into three groups: basic, neutral, and acidic, according to isoelectric- or chromato-focusings (Sugioka et al., 1985; Hayes et al., 1986a,b). Recently, based on similar properties in structure and catalysis shared by major

subunits, GSTs were grouped into three classes: α, μ, and π. This classification is species-independent (Mannervik et al., 1985) and reflects the evolutionary relationship between species.

Placental glutathione S-transferase (GST-P), a neutral form of GSTs with a m. wt. of 23,307, was first purified from rat placenta by Sato et al. in 1984. Of particular relevance to rat GST-P (neutral) are human placental form of GST (GST-π, acidic) and mouse GST M II (basic). All these three GSTs belong to π class of GSTs according to the species-independent classification (Mannervik et al., 1985), share many properties and are immuno-crossreactive to each other (Sato, 1989). For example, the anti-rat GST-P antibody has been found to be cross-reactive in many species such as mouse, hamster, dog, horse and human (Moore et al., 1985; Roomi et al., 1985a; Zhang & Mock, 1992).

1. Structure

The identified forms of GSTs are composed of homodimer or heterodimer subunits (isoforms). The dimers may be separated, in order of their molecular weights, into monomers by means of electrophoresis (Bass et al., 1977; Kitahara et al., 1983a; Satoh et al., 1985) or chromatography (Ketterer et al., 1987; Ostlund-Farrants et al., 1987).

2. Metabolic roles

As mentioned above, GSTs are intimately related to GGT in their metabolism as both GSTs and GGT participate in mercapturic pathway metabolism and therefore in the detoxification of

electrophilic compounds such as carcinogens. In addition, certain forms of GSTs have selenium-independent GSH peroxidase activity toward lipid peroxides via activating P-450 (Kitahara et al., 1983b; Meyer et al., 1985; Ketterer et al., 1987). Furthermore, Ligandin, a basic form of GSTs, and several other form of GSTs, has been shown to function as binding or carrier proteins for a wide spectrum of exogenous materials such as dyes, cholic acid, steroid hormone, hematin, leukotriene and carcinogens (Smith et al., 1977; Jakoby, 1978; Chasseaud, 1979; Ketterer et al., 1985; Mantle et al., 1987).

3. GST-P and carcinogenesis

GST-P, together with GGT, has been found to be the best tumor markers in the rat liver model (Cameron et al., 1978; Ogawa et al., 1980; Hsu et al., 1981; Moore et al., 1987). Among hepatocarcinogens tested, with the exception of the peroxisome proliferator group of hepatocarcinogens (Numoto et al., 1984; Rao et al., 1984, 1986a,b, 1987a,b, 1988; Goel et al., 1986; Glauert et al., 1986; Greaves et al., 1986; Hendrich et al., 1987; Wirth et al., 1987), GST-P and GGT mark more altered hepatic foci than any other hepatic tumor markers (Ito et al., 1988). GST-P(+) foci appear early during hepatic carcinogenesis and have been detected immunohistochemically within 48 h following a single dose of a carcinogen (Moore et al., 1986; Sato, 1988). The staining persisted for at least 6 months, suggesting an irreversible property (Takahashi et al., 1987; Sato, 1988). The number of the positive cells in the foci is proportional to the increase in the dosage of a carcinogen (Moore et al., 1987a).

There is evidence indicating that GST-P may be a better tumor marker than GGT in hepatic carcinogenesis. Unlike GGT, GST-P is not inducible by administration of a large variety of promoters and other drugs (Roomi et al., 1985b; Satoh et al., 1985; Ito et al., 1988), although it is slightly inducible by some antioxidants (Tatematsu et al., 1985, 1987, 1988b) and by ethoxyquine (Thamavit et al., 1985; Manson et al., 1987) in periportal areas. Such weak activity does not interfere with detection of GST-P foci. However, GGT can be induced so strongly by various promoters and drugs that the enzyme-altered neoplastic foci are no longer recognizable (Tatematsu et al., 1985; Fischer et al., 1986). GST-P staining scores more altered hepatic foci than GGT staining (Tatematsu et al., 1985). While GGT staining is rapidly lost following withdraw of carcinogen treatment (Moore et al., Tatematsu et al., 1988a,b), GST-P staining is relatively stable (Sato, 1989).

GST-P was also found to be elevated in preneoplastic and neoplastic lesions in a number of experimental models (Moore et al., 1985). and in human organs such as colon (Kodate et al., 1986; Peters et al., 1989), uterine cervix (Shiratori et al., 1987), esophagus and stomach (Tsutsumi et al., 1987), liver (Sato et al., 1987), kidney (Shea et al., 1987), and lung (Nakagawa et al., 1988). Recently, GST-P has been found to be a potential tumor marker in the HBPM model (Zhang & Mock, 1992).

The GST-P gene is located on chromosome 1q43 (Masuda et al., 1986) and is associated with cis-acting regulatory elements (Okuda et al., 1987). It has been found that both up and downstream enhancers of GST-P gene contain sequences which resemble that of

TPA response element. The activation of GST-P gene has been reported to involve AP-1 production which functions in trans-activation (Okuda et al., 1988; Sakai et al., 1988). The GST-P gene expression is shown to be related to activation of oncogenes, such as Ha-ras (Power et al., 1987), metallothionein-ras (Li et al., 1988), SV 40 and Jun (Imler et al., 1988). Because GST-P mRNA level is very low in normal tissue and may be modified in the promotion stage, the expression of GST-P in neoplasia is considered to be a result of gene altaration or regulation at transcriptional level (Muramatsu et al., 1987).

While the role of GST-P in carcinogenesis is not clear, a number of possible roles have been suggested. As discussed above, similar to GGT, it has been proposed that the higher level of GST-P in preneoplastic and neoplastic cells may have better capacity in detoxification, resulting in a selective growth advantage required for further transformation. GST-P is known to possess selenium-independent glutathione peroxidase activity toward lipid peroxides via activation of P-450 (Kitahara et al., 1983b; Meyer et al., 1985; Ketterer et al., 1987). It has been suggested that GST-P expression may be related to the inhibition of lipid peroxidation which has been considered to play an important role(s) during tumor promotion. The clearance of lipid hydroperoxides involves a series of conjugation steps initiated by GST-P-dependent GSH peroxidase. Thus a chain of reactive factors such as reduced GSH, NADPH and G6PD is en bloc increased (Deml & Oesterle, 1980; Kitahara et al., 1983a,b; Sato et al., 1987).

4. Tissue distribution

A number of studies have investigated the distribution of GST-P in the tissues of adult rats, mice and humans, but few studies have dealt with fetal tissues.

In rats, the protein content of GST-P is generally low or absent in normal tissues, including placenta, fetal lung and livers, adult lung, livers, regenerating livers, heart, testis, prostate, spleen, muscles, and is significantly high in kidney and pancreas (Satoh et al., 1985; Sato, 1989). Using immunohistochemical methods, strong GST-P staining is found in adult kidney tubular epithelium (Tsuda et al., 1985), pancreas ductular cells (Moore at al., 1985), small intestine columnar epithelium (Mannervik et al., 1987), skin epidermis, lung bronchiolar epithelium (Yamamoto et al., 1988) and brain astroglia cell (Tsuchida et al., 1987). GST-P is negative in hepatocytes, but weakly positive in bile ductular cells and placenta.

In the mouse, GST MII, corresponding to class π GST, is found in significant amounts in the livers of male adult mice but are low in females. No GST-P can be demonstrated in other organs, including heart, lung, kidney, intestine, gall bladder and skin. Skin papillomas show no GST-P staining (Roomi et al., 1985a).

In humans, the corresponding GST-π or human GST-P is present normally in the epithelial cells of a wide variety of tissues and organs in contrast to the limited tissue distribution of GST-P in the normal rat and its absence in almost all tissues and organs in mice, except in male mouse livers. In human fetus, GST-P is the main or the only isoenzyme in placenta, lung, kidney, brain and intestine (Koskelo et al., 1981; Polidoro et al., 1982; Koskelo, 1983; Pacifici et

al., 1986, Shiratori et al., 1987). A high level of GST-P is found in fetal liver (Mannervik 1979; Polidoro et al., 1980; Warholm et al., 1980; Koskelo et al., 1981) and stomach (Tsutsumi et al., 1987). In adults, GST-P is the major type of GSTs in lung, brain and spleen (Polidoro et al., 1982; Pacifici et al., 1986, Koskelo et al., 1981; Koskelo 1983). GST-P is abundant in human skin epidermis (Konohana et al., 1990). It can be detected also in adult breast (Ilio et al., 1986), kidney (Ilio et al., 1987), and intestine (Peters et al., 1989), and it is absent in the liver (Van der Jagt et al., 1985; Roomi et al., 1985a; Soma et al., 1986).

Using immunohistochemical methods, Tsutsumi et al., (1987) showed that GST-P strongly stained the surface mucous cells and glands located in the fundic, pyloric and cardiac areas of the stomach of a human fetus aged 18 weeks. The staining decreased as the fetuses aged and no staining was evident in the 34-week-old fetuses. The adult stomach showed only slight staining in the parietal cells of fundic glands.

Very few studies have dealt with the normal distribution of GST-P in hamsters. Using rabbit anti-rat GST-P antibody, Roomi et al. (1985a) found that the liver cytosol of most animals, including hamsters, showed no GST-P activity, while horse and mouse liver cytosol reacted. In the course of studying hamster pancreatic and hepatic carcinogenesis, Moore et al., (1985) observed no GST-P staining in hamster liver and pancreas. GST-P activity has not been investigated in other hamster organs and tissues, including pouch mucosa, salivary gland parenchyma and the odontogenic apparatus.

The normal distribution of GST-P in oral mucosa, salivary glands and odontogenic tissues has not been studied in any species.

As mentioned above, GST-P has been found to be a useful tumor marker, mainly in a variety of epithelial preneoplastic and neoplastic lesions in a number of experimental animals and in humans. The increase or decrease of GST-P in neoplasia seems to be in reverse relationship with the normal distribution of GST-P in tissues and organs. As summarized by Sato (1989), in cell types normally expressing large amounts of GST-P, such as human and rat kidney tubular epithelium, a decrease has been noted during carcinogenesis (Tsuda et al., 1985; Di Ilio et al, 1987; Kurata et al., 1987;), and often an increase is observed in organs normally not expressing GST-P, such as acinar cell lesions of the rat and hamster pancreas and in squamous metaplasias and squamous cell carcinomas in the rat lung. In rat colon carcinoma, GST-P is not expressed, in contrast to positive findings reported for human colonic adenomas and carcinomas. The normal human colon is negative for GST-P (Kodate et al., 1986).

The increase or decrease of GST-P in neoplasia seems to be oncofetal in nature in a number of organs, such as human and rat colon, and human kidney, liver and stomach. In some other organs, however, GST-P is not an oncofetal marker.

EXPERIMENT

As discussed above, there is no potent tumor promoter found in the HBPM model, although weak promoting agents such as benzoyl peroxide have been established. Tumor promotion is an important step in carcinogenesis. The lack of an effective promoter has been the biggest obstacle in exploring many fundamental aspects of carcinogenesis using the HBPM model.

Experiments I and II were designed to explore the tumor promotion potential of two new tumor promoters, OA and MMS, on HBPM. Since OA is extremely expensive, only a pilot study was designed (Experiment I) to investigate its irritating effects on HBPM. It is well know that most, if not all, tumor promoters of skin and mucosa are irritants, although the reverse is not true (Shubik, 1950).

GGT and GST-P have been found to be increased during HBPM carcinogenesis. Whether such increases are oncofetal remains to be answered. No study has investigated GGT and GST-P expression in fetal or neonatal hamster pouches or hamster oral mucosae. There is only one study that investigated the normal tissue distribution of GGT in hamster tissues. There are no data available regarding the normal tissue distribution of GST-P in hamster tissue with exception of adult hamster liver and pancreas. Experiment III was designed to investigate the normal tissue distribution of GGT and GST-P during the development of hamster pouches and several selected organs and tissues. The results should improve our understanding of the normal distributions and functions of GGT and GST-P in hamster tissues and organs, and of the enzymatic increase in neoplasia of the model.

Experiment I: In vivo mitotic activity of okadaic acid on hamster buccal pouch mucosa

1. Objective

To study the effect of OA on hamster buccal pouch epithelium through gross observation and mitosis assay.

2. Materials and methods

Animals

Non-inbreeding male Syrian hamsters (Charles River Breeding Laboratories, MA) aged 8-9 weeks and weighing 105-135 g were used. Animals were fed a commercial stack diet (Puria Formula Chow) and tap water ad libitum and maintained in the standardized conditions of temperature and humidity with a 12 h light/dark cycle (06:00-18:00 light).

OA dosage determination

Generally, the doses of test carcinogens used in the HBPM model are higher than those used in the mouse skin model. Since a dosage of 10 μg OA in 0.1 ml acetone was used in the mouse skin model for tumor promotion (Suganuma et al., 1988), concentrations of 20 μg and 10 μg OA in 0.1 ml acetone were chosen for the dosage trial.

Both pouches of two hamsters were treated once with 20 μg or 10 μg OA (Sigma Chemical Company, St. Louis, MO) in 0.1 ml acetone and sacrificed the second day. All pouches showed obvious inflammation with marked edema, erythema and focal ulceration.

Because ulceration was much more severe on the pouches treated with the higher concentration of OA, it was determined that the lower concentration would be used for the study.

Animal treatment

Six hamsters were randomized into two groups with three animals in each group. Animals were anesthetized by carbon dioxide inhalation. Buccal pouches were cleaned with tap water and dried with gauzes. 10 μg OA in 0.1 ml acetone (stored at -20°C) was applied topically on the surface of a pouch with the aid of a micropipette. The pouch was then inserted back into the hamster's mouth after the acetone had almost evaporated. Both pouches of the three experimental hamsters were treated with OA. In the control group, the right pouches of hamsters were treated similarly with 0.1 ml acetone (also stored at -20°C), and the left pouches were left untreated.

Mitosis assay

The method used to assay mitoses was described by Scragg and Johnson (1980) and slightly modified.

Nineteen and a half hours after the treatment, all animals were given 0.1% vinblastin (VLB, Sigma Chemical Company, St. Louis, MO) intraperitoneally at a dose of 4 mg/kg body weight (Thilagaratnam and Main, 1972). All injections were performed between 08:30 and 09:30 in order to minimize the influence from diurnal variation (Scragg & Johnson, 1980). The animals were then sacrificed 4.5 h later (24 h after OA application) by carbon dioxide inhalation. The

pouches were surgically removed and examined grossly. A longitudinal strip of pouch mucosa approximately 10 mm wide was dissected from the underlying muscle along the entire length of a pouch wall and rolled onto a disposable needle in an anteroposterior direction to form a compact cylinder. Two strips of pouch mucosa per pouch were collected.

Specimens were fixed in 10% buffered neutral formalin for one week and then underwent normal paraffin processing after the needles were removed. After the rolled edge of a mucosa cylinder was embedded at right angles to the block face, three 5 μ sections separated by at least 500 μ were taken from each roll. Hence six sections were sampled from each pouch. The slides were stained with haematoxylin and eosin.

All 72 sections were examined with a light microscope at X400 magnification. The number of arrested metaphase figures in three thousand basal cells per section was recorded. Student *t* test was employed for statistical analysis.

3. Results

Gross and microscopic

The control pouches, both untreated and acetone treated, were unremarkable (Fig. 1.1a). OA-treated pouches appeared heavily inflamed. Redness, edema, petechiae, ulceration and shrinkage of pouch walls were obvious (Fig. 1.1b).

On microscopic examination, OA-treated pouches demonstrated acute inflammation with numerous dilated blood vessels, ulcers and a heavy infiltration of neutrophiles.

Mitosis assay

The blocked metaphase figures were ball-shaped or wreath-like with condensed clumped or annular chromatin (Fig. 1.2). Mitosis index (MI) expressed as number of metaphase figures per 100 basal cells was considerably elevated in OA treated pouches (5.9±1.0) as compared with those of two controls ($p<0.01$). There was no significant statistical difference in MI between the untreated (4.36±0.72) and the acetone treated (4.11±0.43) control pouches ($p>0.05$) (Table 1.1).

4. Discussion

Although the phorbol ester group of tumor promoters, especially TPA, are potent tumor promoters on mouse skin (Van Suuren, 1969), phorbol esters have no reliable promoting effect on hamster buccal pouch epithelium (Silberman & Shklar, 1963), possibly due to the existence of enzymes that rapidly inactivate phorbol esters (O'Brien & Diamond, 1979). Study of the initiation-promotion mechanism of carcinogenesis in the HBPM model has been hampered, owing to a lack of accepted tumor promoter to carry out the promotion procedures, so far routinely performed by a complete carcinogen DMBA. As a result, the subsequent work in exploring a preneoplastic marker for the HBPM model is inevitably hindered.

There are two reasons for choosing OA for the study. The first consideration in choosing a potential tumor promoter for this experiment is that it has been proved in other systems to be a tumor promoter. The second consideration is that the potential tumor promoter should be structurally and mechanistically different from

the TPA-type tumor promoters. OA fulfils both criteria: it is a potent tumor promoter in the mouse skin model (Suganuma et al., 1988), and it has a different chemical structure and functional pathway as compared to the phorbol ester group of promoters, as OA does not react with phorbol ester receptors.

The results of this study showed that OA produced marked inflammatory effects after only a single treatment at a dose (10 μg) similar to that used in the mouse skin model (Suganuma et al., 1988). The mitotic activity of OA-treated pouches was significantly higher than those of the controls, indicating that OA stimulated pouch epithelial cell proliferation. Such properties are essential features for most, if not all, skin and mucosa tumor promoters (Shubik, 1950). Long term study is needed to investigate the possible tumor promoting effects of OA on carcinogenesis in the HBPM. In contrast, teleocidin, a TPA-type mouse skin tumor promoters, showed no irritating effect on HBPM after 8 weeks of topical treatment (unpublished data, L. Zhang) with a dosage (15 μg) 6 times the dosage used in the mouse skin model (Suganuma et al., 1988). If OA proves to be a potent tumor promoter in the HBPM model with long term study, it would seem that HBPM is resistant to TPA and TPA-type of tumor promoters in general, but may respond to non-TPA-type promoters.

Experiment II: In vivo tumor promoting effect of methylmethanesulfonate (MMS) on hamster buccal pouch mucosa

1. Objective

To test the potential tumor promoting effects of MMS in the HBPM model.

2. Materials and methods

Animals

As in experiment I.

MMS dosage determination

Since the promotion dosage of MMS used in the mouse skin model is 10% (100 μmol, Fürstenberger et al., 1989), and since hamster pouch mucosa, in general, requires higher a dosage of chemicals than the mouse skin model, we first tested the effects of MMS in the HBPM medel using the same or higher concentrations. The right pouches of 3 hamsters were painted with 10%, 20%, or 30% MMS in acetone (stored at -4°C) respectively. The left pouches were treated with acetone similarly and was used as controls. In 24 h, animals were anesthetized by carbon dioxide inhalation and pouches were pulled out and examined with the naked eye. All MMS treated pouches showed very strong inflammatory responses in the form of severe edema, petechiae and ulceration. It was decided that such strong irritating effects would not be tolerated by hamsters in a long term study. Subsequently we tested the effects of lower

concentrations of MMS on HBPM. The right pouches of 6 hamsters were painted with 1%, or 5%, or 10% of MMS. In 24 h, the hamsters were anesthetized and the pouches were examined. Pouches treated with 5% and 10% of MMS showed strong inflammatory responses while pouches treated with 1% MMS demonstrated only mild inflammation, which might be tolerated by hamsters in a long term study. Therefore, the concentration of 1% MMS was chosen for a longer term test. The right pouches of 6 hamsters were painted with 1% MMS triweekly. After 2 weeks of promotion, half of the animals showed bleeding from mouth and anus, and muscle stiffness. One animal demonstrated decerebrate rigidity, and all animals looked sick. It was finally decided that a 0.5% MMS would be used in the long term study.

Animal treatment

In a study of the ideal tumor initiating dosage for the HBPM model, McGaughey et al. (1984) found that treating hamster pouches triweekly with 0.2% DMBA for two weeks was the best initiating protocol as such treatment did not produce any changes without further treatment but yielded highest tumor incidence with subsequent applications of a tumor promoter. Therefore, it was decided that this initiating protocol would be used in this experiment. Twenty-five hamsters were divided into 3 groups and subjected to the following treatment (Table 2.1):

Group I (DMBA + MMS or nothing): Both pouches of 10 hamsters were painted with 0.2% DMBA (Sigma Chemical Company, St. Louis, Mo.) in acetone triweekly for 2 weeks, and left untreated

for 10 weeks. The right pouches then were promoted with topical 0.5% MMS biweekly and the left pouches were left untreated.

Group II (MMS + MMS or nothing): Both pouches of 10 hamsters were painted with 0.5% MMS triweekly for 2 weeks, and left untreated for 10 weeks. The right pouches then were promoted with 0.5% MMS biweekly while the left pouches were left untreated.

Group III (DMBA or MMS + acetone): The right pouches of 5 hamsters were painted with 0.2% DMBA, while the left pouches were painted with 0.5% MMS triweekly for 2 weeks. The animals then were left untreated for 10 weeks and subsequently promoted with acetone biweekly.

During the promotion period, the animals were anesthetized with carbon dioxide inhalation, and the pouches were examined periodically for the appearance of tumors. After 10 weeks of promotion, four tumors appeared in the Group I animals. It was decided to terminate the experiment at this time and all the animals were sacrificed by carbon dioxide inhalation.

Samples of skin and hair were excised from hamsters with hair discoloring in Group I and from control hamsters. All pouches were excised and examined grossly. Both the pouch and skin specimens were fixed in 10% formalin for 1 week, and subsequently processed and embedded in paraffin wax. Five μm sections were cut, stained with hematoxylin and eosin and examined under a light microscope.

3. Results

Gross

Group I (DMBA + MMS or nothing): Muscle stiffness and a change of fur color from dark brown to light grey were noted in three of the ten hamsters (Fig. 2.1). At the end of the experiment, all the animals appeared ill with weight loss as compared to the other groups. Two of the ten hamsters demonstrated a total of four tumors on the DMBA-initiated, MMS-promoted right pouches. The tumors appeared either smooth surfaced or cauliflower-like (Fig. 2.2). The color of the tumors varyed from grayish to reddish. The size of the tumors ranged from 3-6 mm in diameter, averaging 4.5 mm. All the DMBA-initiated, MMS-promoted right pouches showed shrinkage and marked thickening of the mucosa, and were erythematous with occasional ulcerations (Fig. 2.2). The unpromoted left pouches were unremarkable.

Group II (MMS + MMS or nothing), Group III (DMBA or MMS + acetone): No obvious toxic effects of MMS, such as decreases in body weight and changes in hair color, were noted in MMS-treated animals as compared to Group I animals (Fig. 2.1). All the pouches looked unremarkable (Fig. 2.2).

Histology

Group I (DMBA + MMS or nothing): There were no microscopically recognizable changes, including the amount of melanin and number of melanocytes, in the skin and hair of hamsters showing gross hair discoloration as compared to the control animals.

Epithelium of the DMBA-initiated, MMS-promoted right pouches showed generalized, marked acanthosis and hyperkeratosis

with frequent down-growth of rete ridges. In many areas, the epithelium was at least three times as thick as the the epithelium in the control groups (Fig. 2.3). Patches of inflammatory infiltration were noted and the inflammatory cells were either primarily chronic inflammatory cells, such as lymphocytes and plasma cells or mixed acute and chronic inflammatory cells (Fig. 2.4c). As shown in Table 2.2, all right pouches showed generalized, moderate dysplasia and 5 of the 10 right pouches demonstrated areas of severe dysplasia (Fig. 2.3b,c). The 4 tumors, from the two hamsters, showed a papillary or pebbly surface and were lined with moderately to severely dysplastic epithelium (Fig. 2.4a). Areas of invasion were noted (Fig. 2.4b).

The DMBA-initiated but unpromoted left pouches showed occasional thickening of the lining epithelium and occasional patches of chronic inflammation. Two of the ten left pouches showed small areas of dysplasia: one pouch from a hamster with tumors on the oposite pouch demonstrated one small focus of moderate to severe dysplastic change and the other pouch revealed two small areas with mild to moderate dysplasia.

Group II (MMS + MMS or nothing): Both the MMS-initiated, MMS-promoted right pouches and the unpromoted left pouches were unremarkable, although occasional rete ridge formations were noted in the right pouches.

Group III (DMBA or MMS + acetone): Both the DMBA-initiated, acetone-promoted right pouches and the MMS-initiated, acetone-promoted left pouches were unremarkable, although occasional

patches of chronic inflammatory cells were noted in the right pouches.

Statistically, DMBA-initiated, MMS-promoted right pouches in group I showed significant differences compared to the controls in terms of tumor yield, tumor rate ($P<0.05$), and dysplasia rate ($P<0.01$) (Table 2.3).

4. Discussion

The results of the study showed that the alkylating agent MMS induced dysplasias and tumors in HBPM initiated with 0.2% DMBA for 2 weeks, and hence it is a tumor promoter in this model. The study also demonstrated that MMS is not carcinogenic in the HBPM model, under the conditions of the study.

Two animals in Group I also demonstrated one or two small areas of dysplastic change in the left pouches that were only initiated with DMBA but not promoted with MMS. In contrast, similarly DMBA-initiated pouches of Group III animals showed no dysplasia. It is possible that the dysplastic changes noted in the left pouches of Group I animals were a result of cross-contamination of MMS from the right pouch treatment or a result of systemic effects of MMS in these animals. Nonetheless, tumors were noted only in the MMS-promoted right pouches and the presence of tumors and dysplastic changes in the MMS-promoted right pouches were statistically higher than those in the left unpromoted pouches in the Group I animals (Table 2.3).

In hamsters initiated with DMBA, MMS produced marked toxic effects, such as a decrease in body weight, general sickness, muscle

stiffness and a change in hair color. Surprisingly, in those hamsters received no DMBA treatment, similar MMS treatment did not produce obvious toxic effects. Therefore, it seems that the toxic effects resulted from combined effects of DMBA and MMS.

The mechanism of MMS tumor promotion remains unclear. The promoting effect of MMS is generally believed to be due to its clastogenic effects (Cerutti, 1982), which include chromosome aberrations, such as chromosome breaking and sister-chromatid exchange (Natarajan et al., 1984).

In summary, this study demonstrated that MMS is an effective tumor promoter but is not a carcinogenic agent in the HBPM model. The mechanism of tumor promotion by MMS probably results from its clastogenic activity. The establishment of a new tumor promoter should prove to be useful in future studies of stage-wise changes, including enzymatic changes, during HBPM carcinogenesis.

Experiment III: Developmental studies on GGT and GST-P in hamsters' fetal, newborn and adult tissues and organs

1. Objective

To study the tissue and organ distribution of GGT and GST-P during hamster development. In particular, to study GGT and GST-P distributions during hamster pouch development in order to find out if the enzymes' induction during HBPM carcinogenesis is oncofetal in nature.

2. Materials and methods

Animals

The maintenance of animals was similar to that in experiment I. Animals were randomly bred. Two non-inbred hamsters from different mothers were sacrificed at day 9, 13, and 15 of gestation and day 1, 3, 6, and 10 after birth, respectively. The same number of adult female animals was used. The following tissues and organs were quick frozen in liquid nitrogen: pouch epithelial cord, oral mucosa, tongue, tooth bud, salivary gland, skin, nasal and sinus cavity, kidney, liver, stomach, intestine, and lung.

Serial sections (10 μm) were cryostat cut and mounted on gelatine coated slides. They were then fixed in cold acetone for 5 minutes. GGT histochemical staining and GST-P immunohistochemical staining were carried out.

GGT staining method

The histochemical demonstration of GGT was performed according to the method described by Rutenburg et al. (1969).

The sections were incubated for 2 h at room temperature in a medium composed of γ-glutamyl-4-methoxy-2-naphthylamide (GMNA, Polysciences Inc., Warrington, PA), glycylglycine free base (Sigma Chemical Company, St. Louis, MO) and Fast Blue BB salt 'Gurr' (BDH Inc., Toronto, Canada). They were rinsed in a sequence of 0.85% NaCl (GIBCO Inc., Grand Island, NY), 0.1 M cupric sulphate (BDH Inc., Toronto, Canada), and 0.85% NaCl again. GGT cleaves γ-glutamyl groups from GMNA and transfers it to glycylglycine. The enzymatically liberated naphythylamine carrying the negatively charged methoxy is bridged with the positively charged diazonium salt present in the mixture to form a copper-chelated azo dye. Since the dye is rapidly formed and is insoluble, it is restricted to the cells with GGT activity so that diffusion artifact is minimal. After completely dried, sections were counterstained in hematoxylin, covered with glycerin as mounting medium, and sealed with nail polish.

Sections from the kidney of an adult normal hamster were used as a positive control.

GST-P staining method

Immunohistochemical demonstration of GST-P was performed using the avidin-biotin-peroxidase complex (ABC) method as described by Hsu et al. (1981). This technique was developed from peroxidase-antiperoxidase (PAP) staining (Sternberger et al., 1970).

Avidin is a 68,000 glycoprotein with four binding sites for biotin. Covalently coupling of biotin to antigens such as immunoglobulin or the peroxidase molecule makes it possible for them to crosslink with avidin. Hence, avidin and biotin serve as a link for each other by which a three dimensional amplification of antigen-antibody reaction is achieved via three steps: biotinylated secondary antibody, avidin, and biotinylated horseradish peroxidase.

The sections were incubated for 30 min in 0.3% H_2O_2 (BDH Inc., Toronto, Canada) in methanol (BDH Inc., Toronto, Canada) to quench the endogenous peroxidase. They were washed then incubated for 30 min with diluted normal goat serum to block non-specific binding sites. After blotting the excess serum from slides, primary antibody (rabbit anti-rat GST-P) at 1:300 dilution was added to sections. Following a 2 night incubation in fridge, sections were incubated in a sequence of diluted biotinylated secondary antibody (goat anti-rabit IgG), ABC reagent, and peroxidase substrate, diaminobenzidine tetrahydrochloride (DAB). The sections were counterstained with hematoxylin and permanently mounted.

For positive control, carcinogen-treated rat liver rich in GST-P(+) nodules was used. For negative control, primary antibody was substituted for phosphate-buffered saline.

3. Results

The results of GGT and GST-P staining are summarized in Table 3.1.

When the hamsters were examined at day 9 of gestation, no organ formation was observed. Sporadic GGT positive cells were

noted, and were mainly distributed around some cavities. No GST-P activity was noted at this stage.

Intra- and para-oral organs

Cheek pouch. The formation of cheek pouch was noted 3 days before birth as an epithelial bud growing inward from the oral epithelial lining (Fig. 3.1a). The epithelial bud grew caudally as an epithelial column as observed on day 1 before birth (Fig. 3.1b) and continued to grow on day 1 and 3 after birth (Fig. 3.1c,d). At day 6 after birth, there was cytodifferentiation characterized by the appearance of keratohyalin-containing cells and cornified cells in the middle layers of the epithelial column. There was also liquefaction of the middle layers of the epithelium, indicating initiation of pouch cavity formation (Fig. 3.1e). At day 10 after birth, a pouch cavity was formed through liquefaction of epithelial cells in the center of the epithelial islands (Fig. 3.1f). Neither GGT nor GST-P activity was noted at any stage of pouch development.

Oral mucosa. The pre-natal, neo-natal and post-natal tongue mucosae showed no detectable GGT. Aggregates of strongly GGT-positive cells, however, were noted in adult dorsal tongue mucosa. Figure 3.2a demonstrates a tongue section and figure 3.2b a tongue mucosa stripping specimen. The strongly GGT positive cells are confined to the connective tissue of fungiform papillae. They are spindle or stellate in shape (Fig. 3.2c). There was also a weak GGT staining in the stratified squamous epithelial cells adjacent to those strongly GGT positive, spindle or stellate mesenchymal cells. No GST-P(+) reaction was recorded in the tongue mucosa. The remaining oral

mucosa was negative for GGT and GST-P at any stage of the development.

Tooth. As the development stage of a tooth varies in different teeth, for a given age of a hamster, the description of GGT and GST-P staining will be based mainly on the degree of enamel organ formation and amount of dental hard tissue formation instead of on the age of the hamsters.

Before and after birth, both the enamel organ and odontoblasts stained for GGT, though the staining of the enamel organ appeared earlier and stronger than that of odontoblasts. Occasional positive GGT staining was observed in dental lamina (Fig. 3.3a), mainly in the area in which a bell shaped enamel organ was forming. When the enamel organ was formed the stellate reticulum and stratum intermedium cells were the first to stain with GGT prior to, and early in, the deposition of dental hard tissues (Fig. 3.3b,c). The GGT staining of these structures gradually weakened and finally disappeared with the increment of dental hard tissues. Ameloblasts and odontoblasts exhibited GGT activity in their secretion stage following the formation of dental hard tissue. Some GGT staining was also noted in the dental hard tissues, including both enamel and dentin, particularly the predentin.

No GST-P(+) reaction was recorded during tooth development.

Ten day-old and adult teeth were investigated for GGT and GST-P activity because of their high calcium content.

Salivary gland. Some acini lobules of minor salivary glands demonstrated weak GGT positive staining from day 1 after birth (Fig. 3.4a) and became stronger on day 3 and day 6 (Fig. 3.4b), while some

other lobules besides those of positively stained glands showed no reactivity. The ducts of minor salivary glands showed ambiguous staining on day 3 and day 6 after birth. Minor salivary glands were not studied for GGT in 10 day-old and adult hamsters.

Parotid glands were examined in 10 day-old and adult hamsters, when the glands could be easily identified and removed. Two large lobules separated by connective tissue were present. One lobule demonstrated positive GGT staining in the acini in both 10-day old and adult hamsters (Fig. 3.4c). The acini of the other lobule were negative for GGT. The ducts in both lobules showed ambiguous staining in 10 day-old hamsters, but were positive in adult hamsters.

No GST-P(+) reaction was recorded in either major or minor salivary glands.

Nasal and sinus mucosa. Very strong GGT activity was noted in the pseudostratified ciliated epithelial lining cells, especially on the side near the lumen and the cilia on day 1, before and after birth (Fig. 3.5a). Moderate GGT staining was present in a similar location at day 3 after birth (Fig. 3.5b). Other days were negative.

No GST-P staining was noted.

Ten day-old and adult nasal and sinus mucosa were not investigated for GGT and GST-P activity.

Extra-oral organs

Skin. The epidermis showed GGT(-) in the early embryonic stage. Few skin appendages were noted in the early embryonic stage and they were negative for GGT. Immediately before birth and after birth as well as in the adult, numerous hair follicles were formed.

The germinal matrix of both external and internal hair root sheaths, located in the deep portion of the hair follicle, became strongly GGT positive. The epidermis remained negative during the time of the study (Fig. 3.1c,d,e & 3.6).

No GST-P(+) reaction was recorded.

Kidney. Strong GGT activity was demonstrated in renal tubular cells, especially the brush borders in all animals after organ formation (Fig. 3.7). The highest staining intensity was noted in the adult kidney.

No GST-P(+) reaction was recorded.

Liver. Focal weak GGT staining was noted in hepatocytes before birth (-3 and -1 days) but negative after birth. Weak GGT staining was noted occasionally in the biliary duct cells before birth and at day 1 and day 3 after birth, but absent in 10 day-old and adult hamsters.

GST-P was negative before birth. A diffuse staining, moderate in intensity, was noted in hepatocytes at day 1 after birth (Fig. 3.8a), but the staining became weak at day 3 after birth (Fig. 3.8b), negative at day 6 after birth (Fig. 3.8c), ambiguous at day 10 after birth (Fig. 3.8d). Moderate staining was noted again in adult hamster (Fig. 3.8e). The biliary duct cells showed weak staining in all hamsters sacrificed after birth, but the number of positive duct cells decreased with age (Fig. 3.8).

Gastrointestinal tract. On both days before birth (-3 and -1 days), the lining epithelial cells of the gastrointestinal tract, especially the villi on the luminal surface, were strongly GGT positive (Fig. 3.9a,b). No GST-P staining was noted before birth.

After birth, it was possible to differentiate stomach from intestine and the staining results of the two organs were as follows:

Stomach. There was no GGT activity detectable in neonatal hamsters. However, the adult stomach, in the region close to esophagus (fundus), moderate GGT staining was noted in the gastric glands (Fig. 3.9c). A weak GST-P staining was observed in the luminal surface of the lining epithelium at days 1, 3, 6 and 10, but absent in adults (Fig. 3.9d).

Intestine. Strong GGT staining was noted in the epithelial lining cells, especially in the luminal surface and the villi after birth (Fig. 3.9 e-i). No GST-P activity was noted.

Lung. There was no GGT staining in the embryonic respiratory system. After birth the lining epithelium of the airways, particularly the cilia of the respiratory epithelium including trachea, bronchi and bronchioles, showed weak GGT staining at day 1, moderate staining at day 3 and 6, strong staining at day 10, and moderate staining in adult hamsters (Fig. 3.10a-e). No alveoli were stained at any stage of lung development.

Weak GST-P staining was noted in the bronchiolar lining epithelium, mainly on the cilia on day 1 after birth (Fig. 3.10f).

4. Discussion

GGT

The expression of GGT in preneoplasia and in neoplasms in several organs and tissues has been shown to be oncofetal in nature while in other organs, it is not. Increased GGT has been observed during hamster pouch carcinogenesis. The results of this study

showed that there was no GGT activity in hamster pouch at any stage of its development. Therefore, the expression of GGT activity during carcinogenesis of HBPM may represents an acquired gene alteration instead of re-expression of a phenotype that is presented in normal embryonic development.

GGT activity was demonstrated in a number of hamster tissues and organs in this study, primarily in epithelial cells. GGT activity was particularly prominent in cells with 'brush borders', such as epithelial cells of the intestinal mucosa, nasal or sinus mucosa, airways, renal tubules and of ameloblasts. The term 'brush border' denotes a specific plasma membrane structure with numerous finger-like processes with a large surface area, and is intimately associated with the transport of carbohydrates, irons and amino acids (Åhlund-Lindqvist & Lindskog, 1985). The results of this study are similar to those of other studies in other species, and support the hypothesis that GGT may participate normally in amino acid transportation.

As mentioned above, one study investigated GGT activity histochemically in a number of adult hamster organs and tissues (Albert et al., 1964). The results from this current study, in general, agree with those from Albert et al.: both studies showed GGT activity in renal tubules, in the lining epithelium of bowels, in the glandular cells and secretory ducts, but no GGT activity in hepatocytes in adult hamsters. While, Albert et al. (1964) found no GGT activity histochemically in adult hamster lung and stomach, the present study showed moderate GGT staining in the fundus region of gastric glands and the lining epithelium of bronchi and bronchioles.

Occasionally, GGT activity was noted also in mesenchymal cells: odontoblasts and some stellate or spindle cells in the lamina propria of fungiform papilla of adult hamster tongue. Although it is obvious that these stellate and spindle cells are mesenchymal cells, their exact cell type is not clear. Their exclusive location in the connective tissue papilla of fungiform papillae seems to rule out the possibility of fibroblasts as fibroblasts are abundant throughout the tongue mucosa lamina propria. The nature of them is not clear.

GST-P

Unlike the wide distribution of GGT during the normal development of hamster tissues and organs, few cells showed GST-P staining. GST-P activity was notably absent in all hamster intraoral organs and tissues, including the hamster pouch. This suggests that the expression of GST-P activity during carcinogenesis of HBPM may represent an acquired gene alteration instead of re-expression of a phenotype that is presented in normal embryonic development.

Only three organs, liver, lung and stomach, showed weak to moderate GST-P activity and all the staining was confined to epithelial cells.

5. Conclusion

1) GGT/GST-P activity was not found during the development of hamster buccal pouch and oral mucosa. Therefore, it seems that the induction of the enzymes in HBPM carcinogenesis is not oncofetal in nature, but tumor-associated;

2) GGT is found in a number of organs and tissues and is located mainly in the epithelial cells, but occasionally in mesenchymal cells; whereas, GST-P is observed in few organs and is only in epithelium.

3) The predominant location of GGT activity in epithelial cells with 'brush borders' supports the hypothesis that GGT normally participates in amino acid transportation.

TABLES

Table 1.1 Mitotic Activity of OA on HBPM

Animal no	Pouch side	Roll no	MI(%)[a] OA	Acetone	none
1	L	1	5.90		5.10
			4.97		4.60
			4.43		4.10
		2	5.20		6.23
			6.10		4.67
			5.97		4.70
	R	1	5.67	3.47	
			3.20	3.63	
			3.70	3.80	
		2	6.37	4.10	
			5.87	3.73	
			7.86	4.13	
2	L	1	5.43		3.97
			6.20		3.80
			5.17		3.67
		2	7.27		5.07
			6.53		4.97
			6.83		4.13
	R	1	5.87	4.73	
			6.20	3.60	
			5.87	4.17	
		2	6.60	4.03	
			7.07	4.20	
			6.27	4.37	
3	L	1	6.23		2.63
			6.80		4.57
			6.30		3.07
		2	6.37		4.40
			6.13		3.67
			4.93		5.07
	R	1	6.93	3.97	
			6.43	4.03	
			4.90	4.23	
		2	5.20	4.03	
			5.97	5.27	
			6.10	4.49	
Pooled data	X		5.91	4.36	4.11
	Sx(±)		1.00	0.72	0.43
	P			<.001[b]	>0.05[c]

[a] Mitotic Index expressed as percentage of metaphase cells in 3,000 basal cells.

[b] Comparison between OA-treated pouches and controls.

[c] Comparison between acetone-treated and untreated pouches.

Table 2.1 Initiation-promotion experimental design

Group	Animal no	Pouch	Initiation (3/w, 2wks)	NT (10wks)	Promotion (2/w,10wks)
I	10	R	0.2% DMBA	none	0.5% MMS
		L	0.2% DMBA	none	none
II	10	R	0.5% MMS	none	0.5% MMS
		L	0.5% MMS	none	none
III	5	R	0.2% DMBA	none	acetone
		L	0.5% MMS	none	acetone

NT: no treatment
DMBA: dimethylbenz(a)anthracene
MMS: methylmethanesulfonate
#/w: times of treatment per week

Table 2.2 Tumor promoting effect of MMS on HBPM

Group	Pouch	Tumor Yield[a]	Tumor Rate[b]	Dysplasia Rate[b]
I	R	4	2(20%)	10(100%)
	L	0	0(0%)	2(20%)
II	R	0	0(0%)	0(0%)
	L	0	0(0%)	0(0%)
III	R	0	0(0%)	0(0%)
	L	0	0(0%)	0(0%)
P value		<.05(χ^2)	<.05(u)	<.01(u)

[a] Number of tumors in each group.
[b] Number of animals bearing nodule or dysplasia in each group.

Table 2.3 MMS tumor promoting results in HBPM

Animal		1	2	3	4	5	6	7	8	9	10
GI right (DMBA+MMS)	Degree of dysplasia[a]	Mo & S	Mo	Mo	Mo & S	Mo	Mo	Mo & S	Mo	Mo & S	Mo & S
	Extent of dysplasia[b]	+++	+++	+++	+++	+++	++	+++	++	+++	+++
	Tumor	3	-	-	-	-	-	-	-	-	1
	↑ in epithelial thickness[c]	+++	+++	+++	+++	+++	++	+++	+	+	+++
GI left (DMBA +NT)	Degree of dysplasia	Mo to S	Mi to Mo	-	-	-	-	-	-	-	-
	Extent of dysplasia	+	+	-	-	-	-	-	-	-	-
	Tumor	-	-	-	-	-	-	-	-	-	-
	↑ in epithelial thickness	-	-	-	-	-	+	-	-	++	-
GII (MMS + MMS or NT)	Dysplasia & tumor	-	-	-	-	-	-	-	-	-	-
	↑ in epithelial thickness	-	-	-	-	-	-	-	-	-	-
GIII (DMBA or MMS + acetone)	Dysplasia & tumor	-	-	-	-	-					
	↑ in epithelial thickness	-	-	-	-	-					

[a] Mi: mild dysplasia; Mo: moderate dysplasia; S: severe dysplasia.

[b] +: 1 or 2 small focal area(s); +++: generalized.

[c] +: slightly increased in thickness; ++: moderately increased; +++: marked increased.

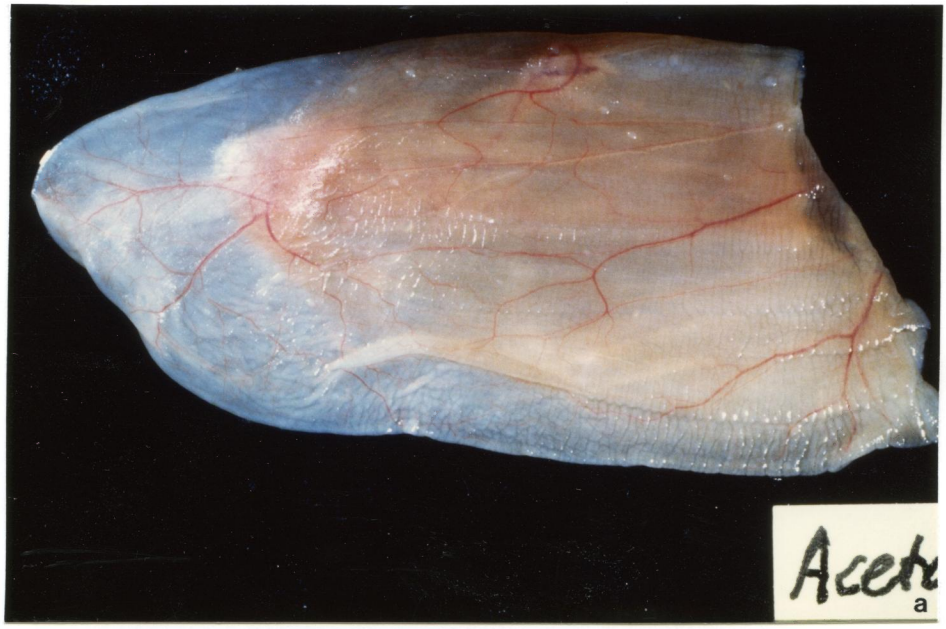
Table 3.1 Normal distribution of GGT/GST-P in developing and adult tissues and organs of Syrian hamster

Organ	Tissues or Cells	Age (Day)						
		-3	-1	1	3	6	10	Adult
Intra- & para-oral organs								
Pouch mucosa	lining epithelia	-/-	-/-	-/-	-/-	-/-	-/-	-/-
Oral mucosa	lining epithelia	-/-	-/-	-/-	-/-	-/-	-/-	-/-
Tongue mucosa	lining epithelia	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	connective tissue	-/-	-/-	-/-	-/-	-/-	-/-	+/-
Tooth								
Dental lamina	epithelia		-/-	-/-	-/-	+/-		
Enamel organ	ameloblasts	-/-	+/-	+/-	++/-	+++/-		
	Stratum intermedium	-/-	++/-	++/-	++/-	+++/-		
	stellate reticulum	-/-	++/-	++/-	++/-	+++/-		
	enamel matrix		-/-	+/-	+/-	+/-		
Dental papilla	odontoblasts	-/-	+/-	+/-	+/-	+/-		
	dentin		-/-	+/-	+/-	+/-		
Salivary gland	acini	-/-	-/-	+/-	++/-	++/-	+/-	+/-
	ducts	-/-	-/-	-/-	±/-	±/-	±/-	+/-
Nasal & sinus mucosa	lining epithelia	-/-	+++/-	+++/-	++/-	-/-		
Extra-oral organs								
Skin	epidermis	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	hair follicles	-/-	++/-	+++/-	+++/-	+++/-	+++/-	+++/-
Kidney	tubular epithelia	++/-	++/-	+++/-	+++/-	+++/-	+++/-	+++/-
Liver	hepatocytes	+/-	+/-	-/++	-/+	-/-	-/±	-/++
	biliary ducts	+/-	+/-	+/+	+/+	+/+	-/+	-/+
Lung	acini	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	bronchial/bronchiol epithelia	-/-	-/-	+/+	++/-	++/-	+++/-	++/-
Gastrointestinal tracts	epithelia	+++/-	+++/-					
Stomach	epithelia			-/±	-/±	-/±	-/++	+/++
Intestine	epithelia			+++/-	+++/-	+++/-	+++/-	+++/-

FIGURES

Fig. 1.1 a) An acetone-treated hamster pouch demonstrating no abnormalities. b) An okadaic acid treated hamster pouch demonstrating erythema, edema, petechia, ulceration and shrinkage of the pouch wall.

Fig. 1.2. A photomicrograph showing numerous ball-shaped, blocked metaphase mitosis figures in the hamster pouch epithelium treated with okadaic acid (H.E., high power view).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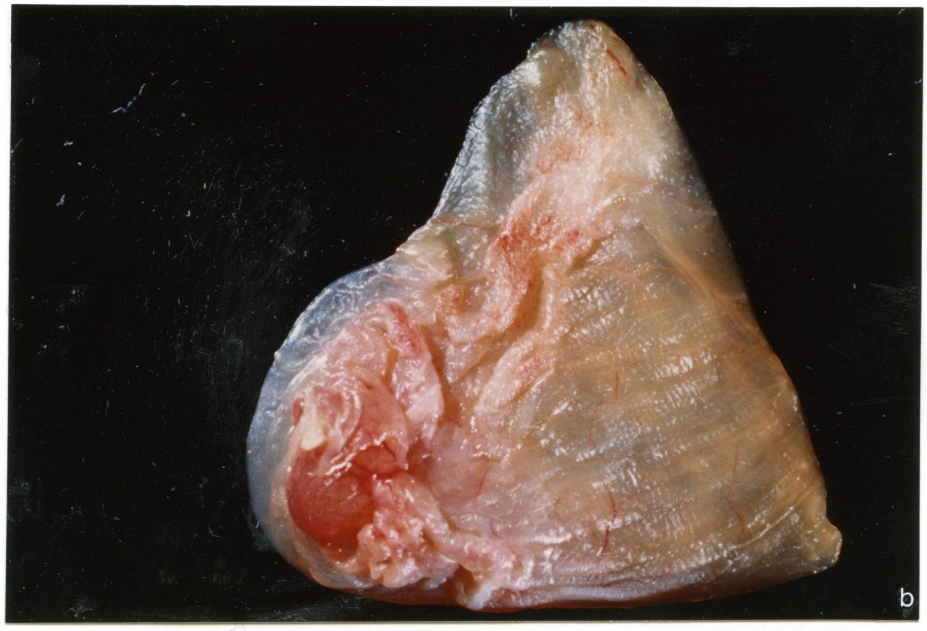
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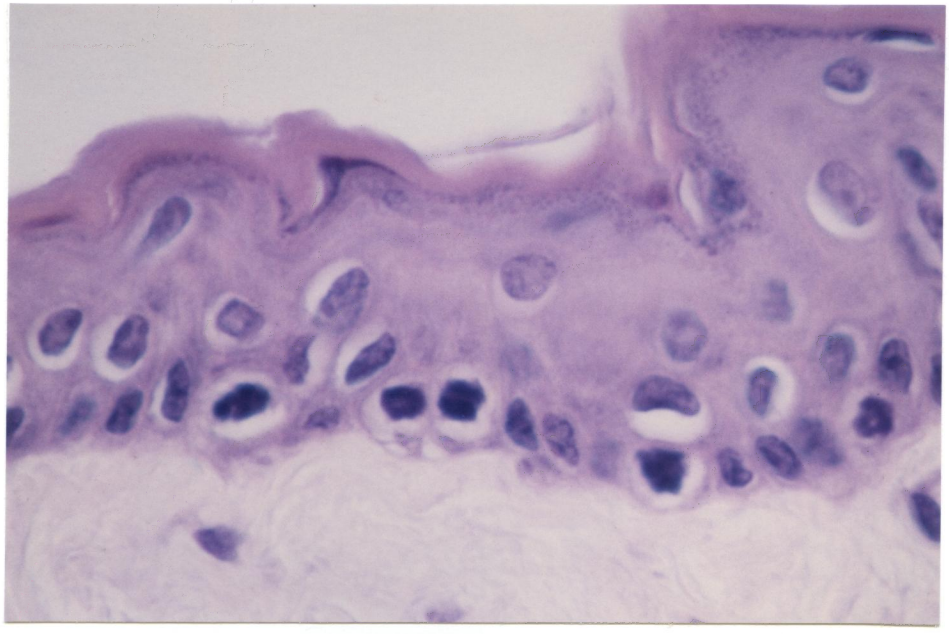
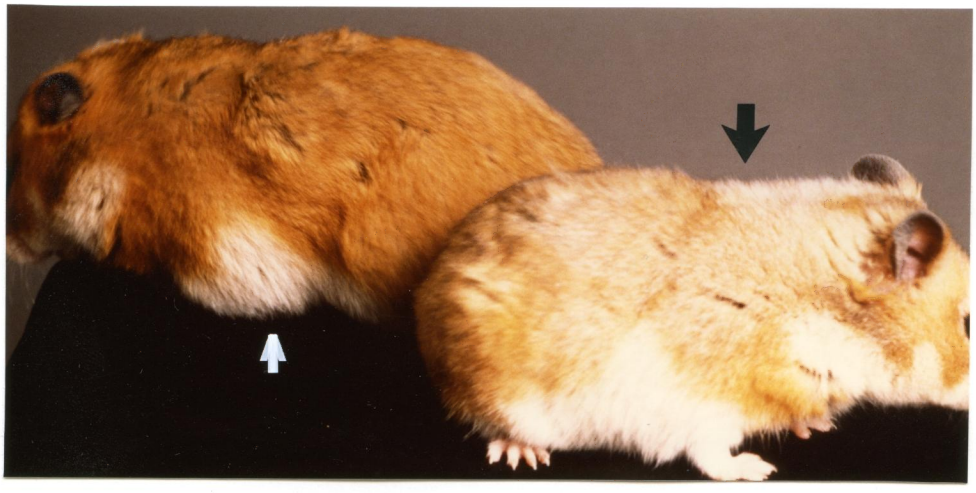


Fig. 2.1. The DMBA-initiated, MMS-promoted hamster (black arrow) demonstrating a change of fur color from dark brown to light grey as compared to the control animal (white arrow).

Fig. 2.2. Tumors (arrows) in the DMBA-initiated and MMS-promoted pouches. The two control pouches on the right side of the picture showing no abnormality.



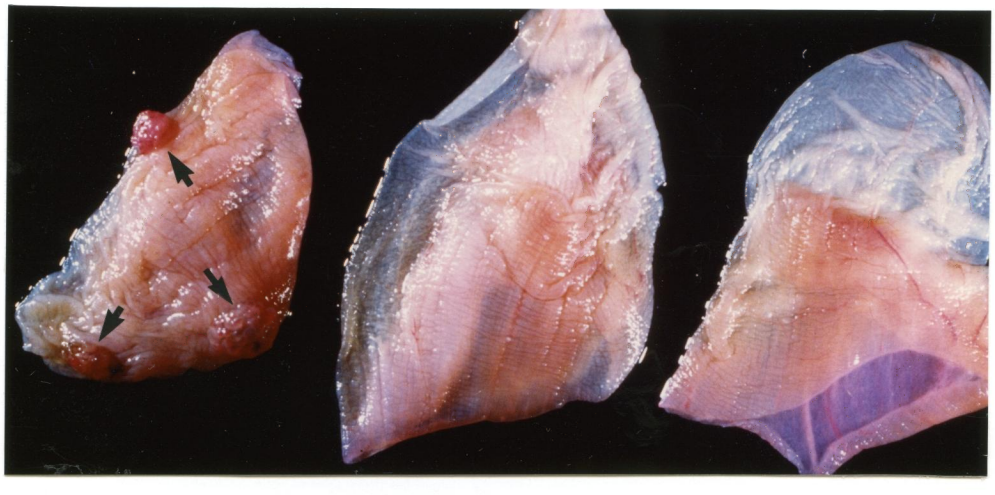
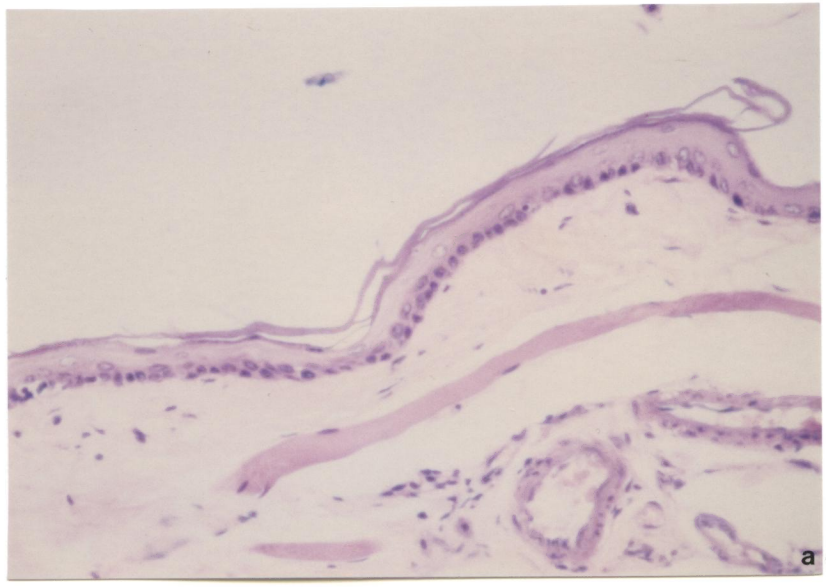


Fig. 2.3. Histological examination. a) Untreated pouch mucosa. b & c) DMBA-initiated, MMS-promoted pouch showing severe dysplasia and marked acanthosis and hyperkeratosis with down-growth of rete ridges. The thickness of epithelium is at least 3 times that of the controls (H-E, high power view).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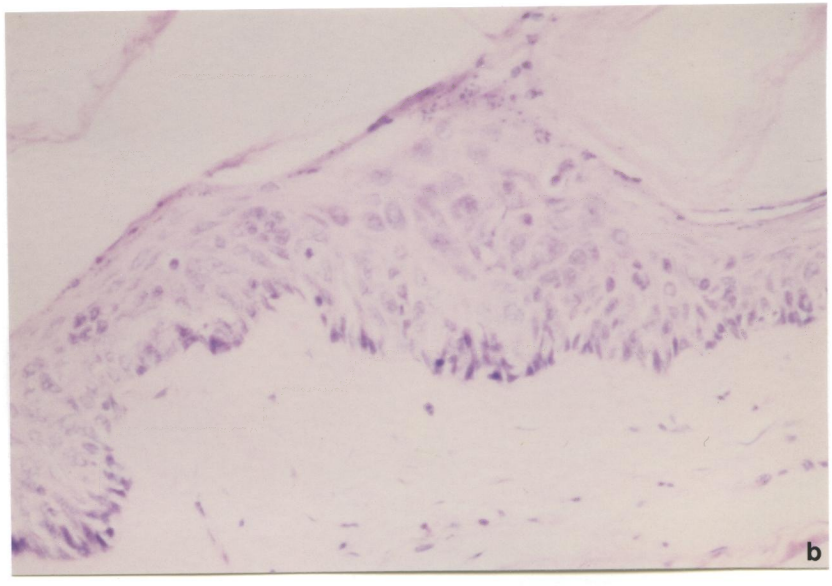
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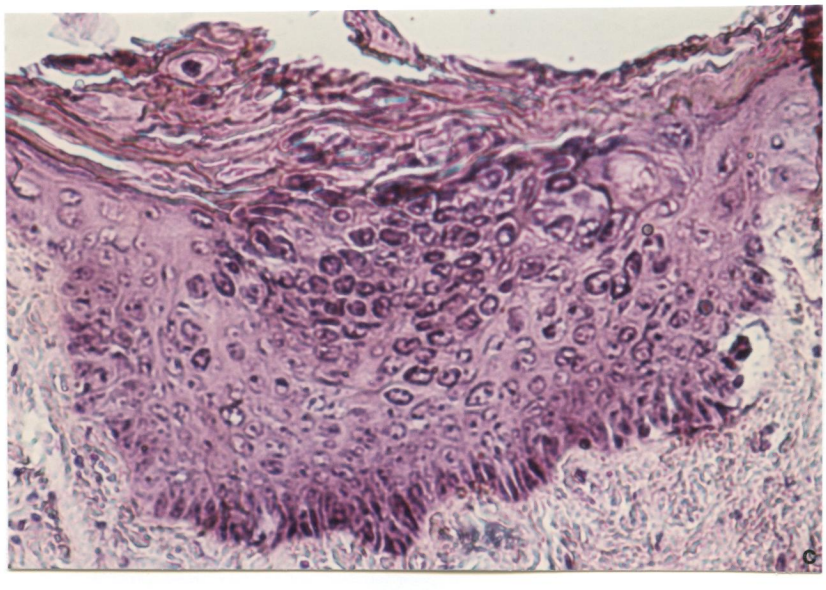
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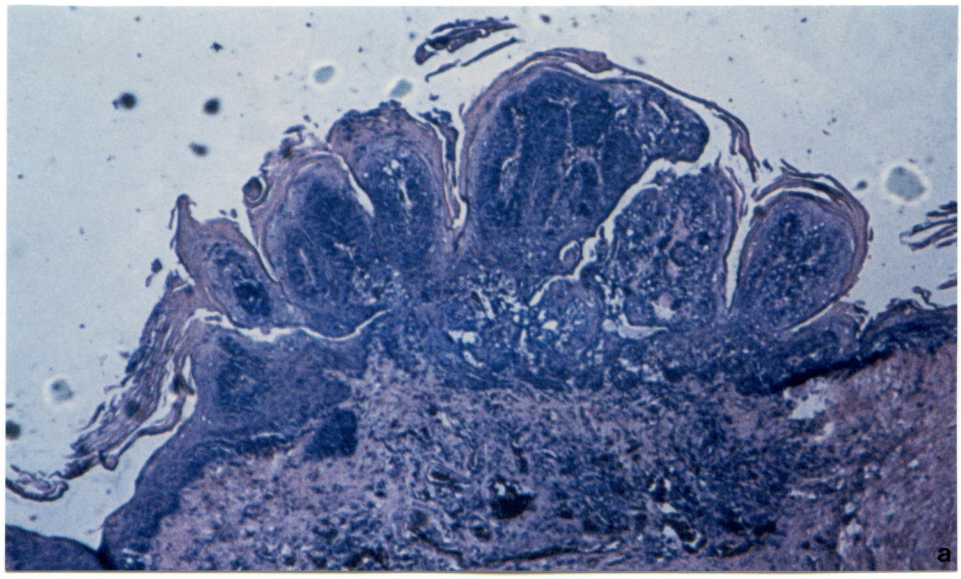
Fig. 2.4. a) A photomicrograph demonstrating a papillary tumor in a DMBA-initiated, MMS-promoted pouch. b) A photomicrograph showing an island of invasive squamous epithelium. c)A DMBA-initiated, MMS-promoted pouch showing a patch of inflammatory infiltration of lymphocytes and plasma cells (H-E, low power view).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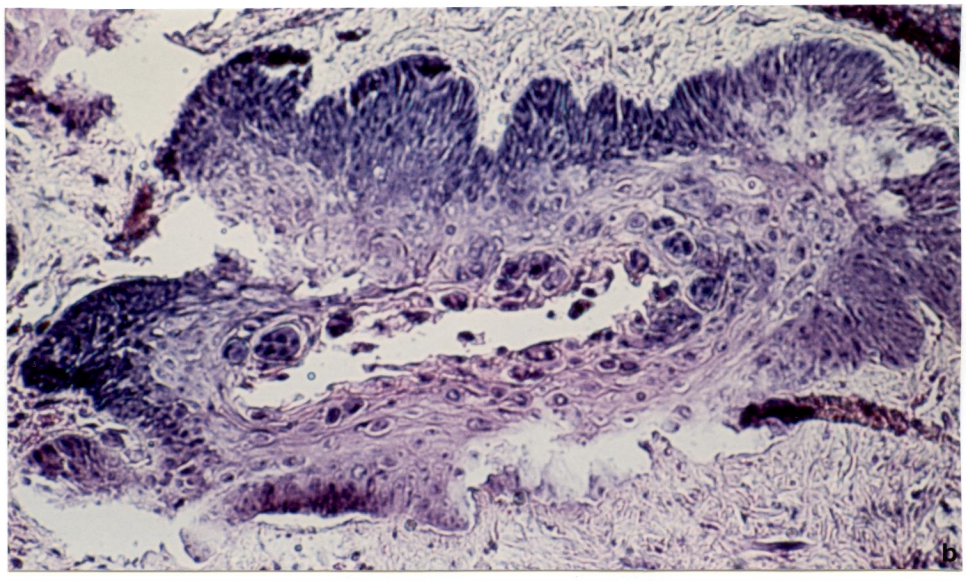
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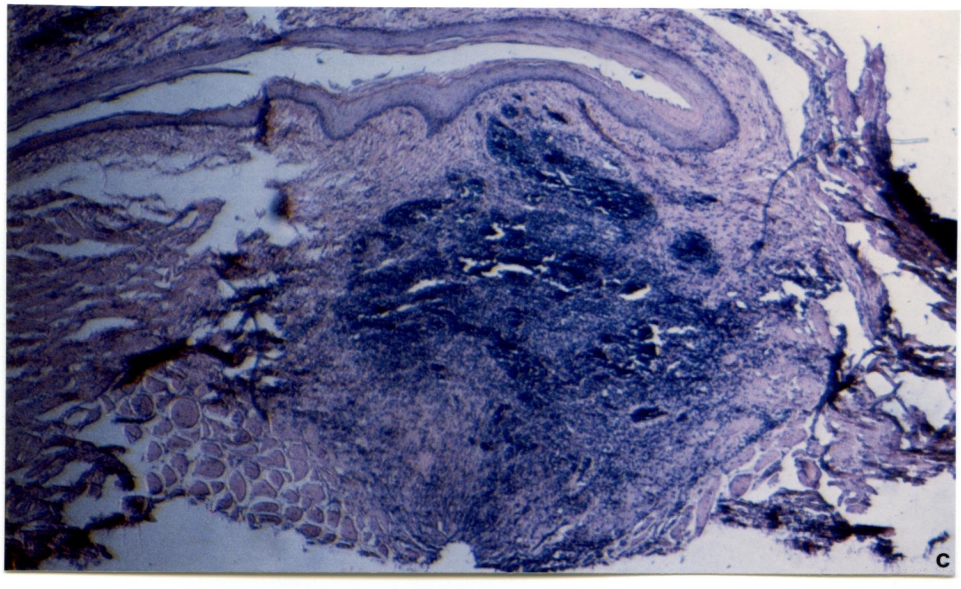
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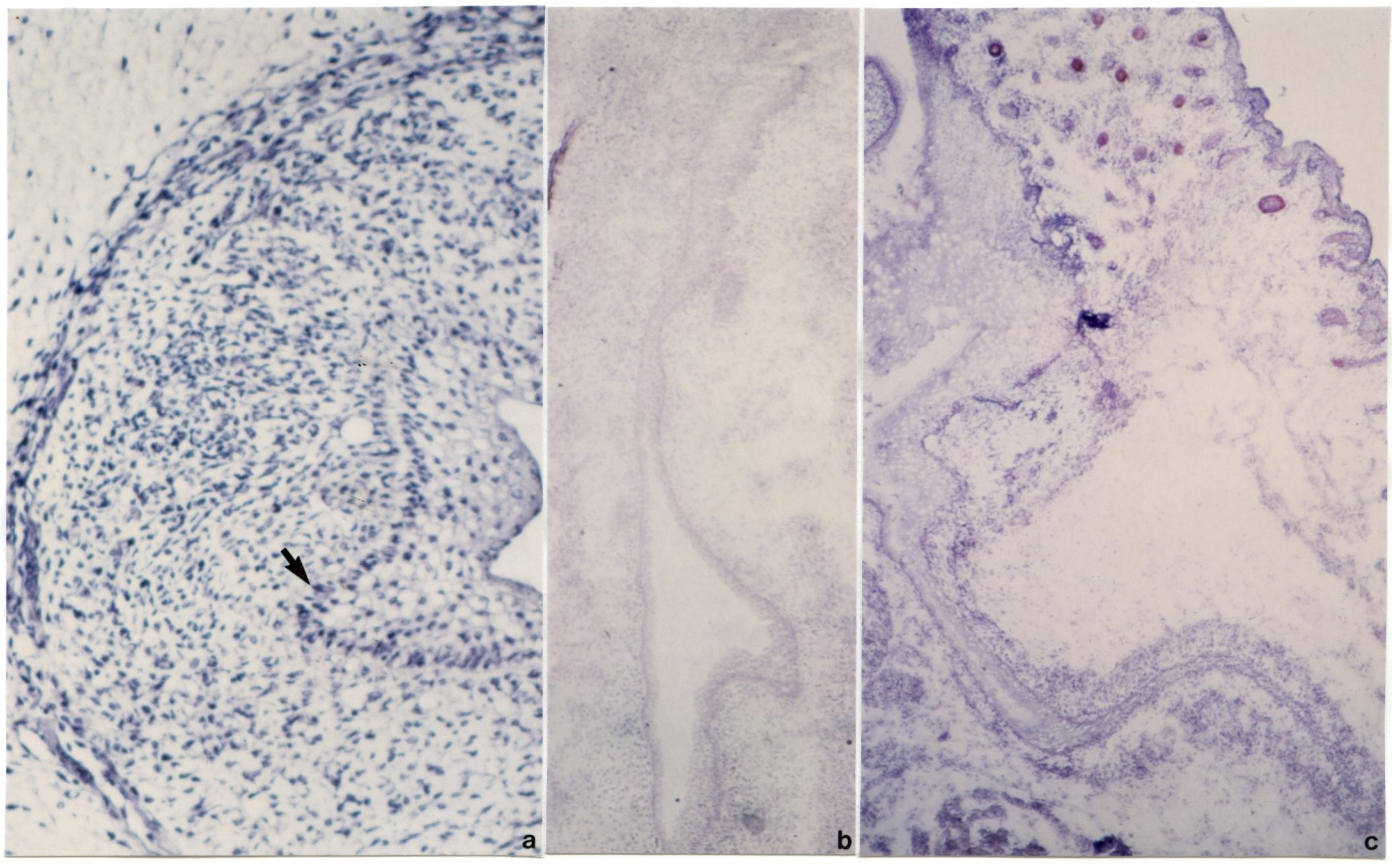
Fig. 3.1. GGT-staining for the development of hamster cheek pouch. a) The formation of the cheek pouch as an epithelial bud (arrow) 3 days before birth. b, c & d) The growth of the bud to form long epithelial cord 1 day before birth, and 1, 3, 6 and 10 days after birth. e) 6-day old new born hamster cheek pouch showing keratohyalin-containing cells and cornified cells as well as liquefaction of these cells in the middle layer of the cord. f) A 10-day old hamster pouch (low power view).

Figures c), d), and e) also show GGT positive staining in germinal matrix of external and internal hair root sheaths.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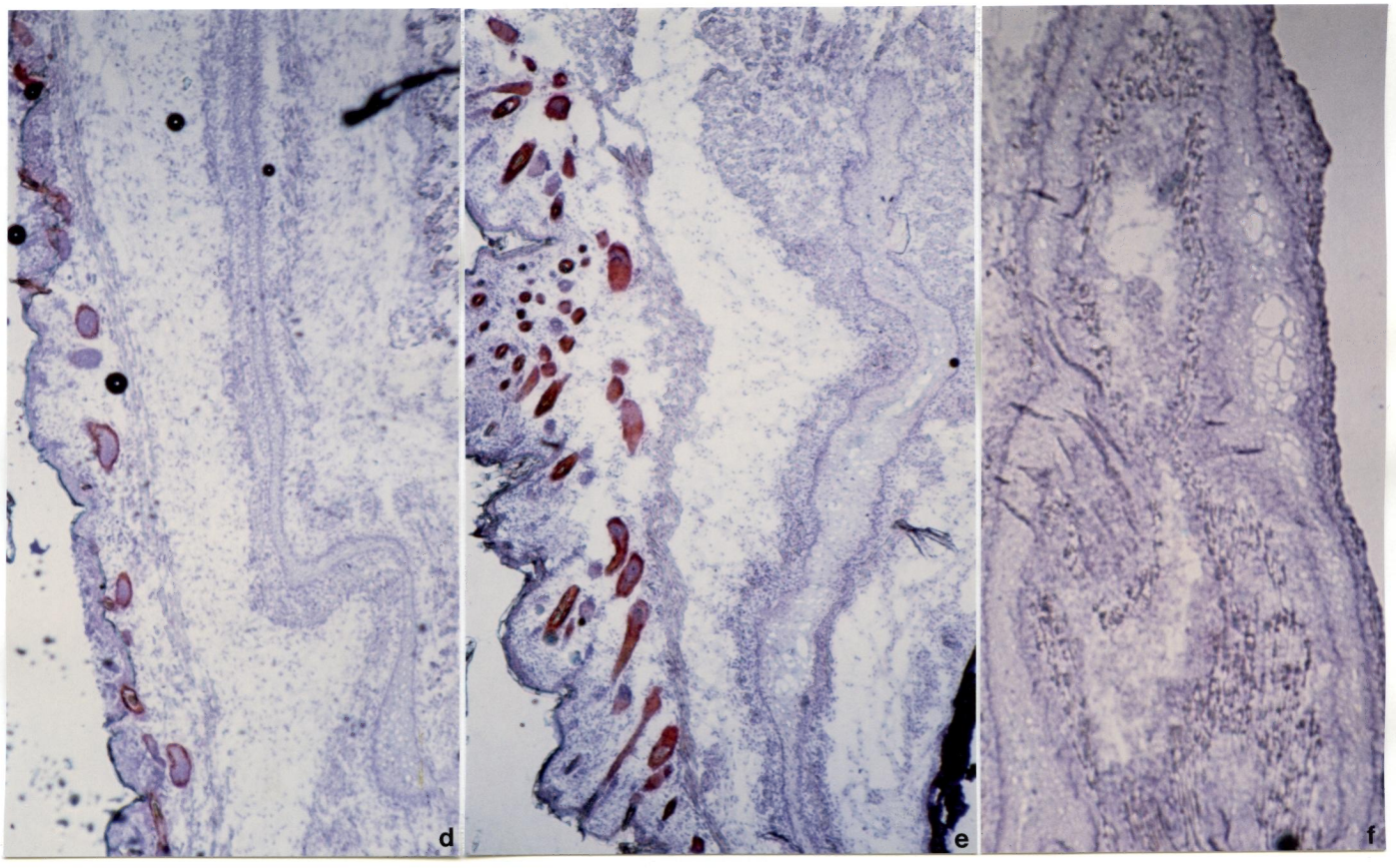
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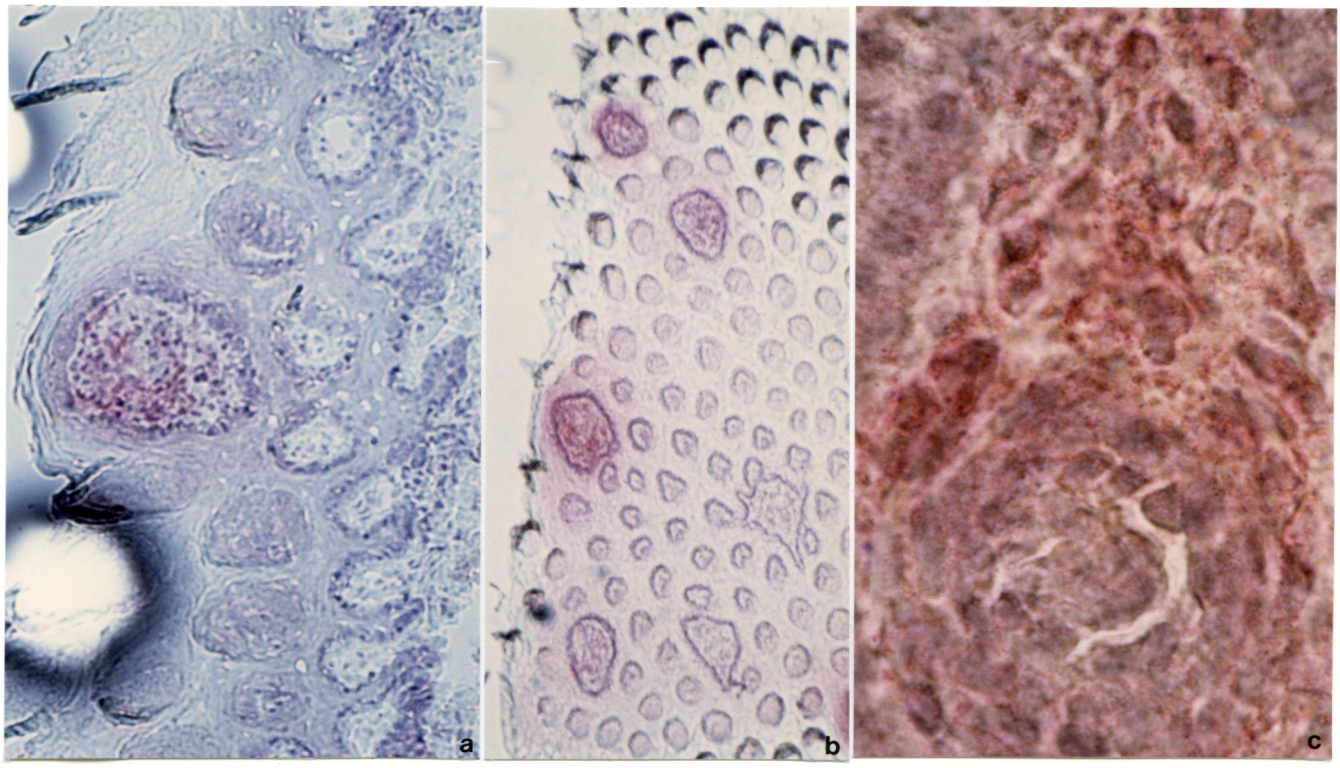
Fig. 3.2. GGT-staining of dorsal tongue mucosa of adult hamster's section and strip specimens. a & b) Low power view showing strong GGT(+) cells in the connective tissue papillae of fungiform papillae (low power view). c) High magnification view of the spindle or stellate shaped GGT positive cells (high power view).

Fig. 3.3. GGT-staining for tooth developing-related structures. a) A photomicrograph showing GGT(+) staining in dental lamina, where a bell-shaped enamel organ was forming. b & c) Photographs showing strong GGT(+) staining in the enamel organs (low power view).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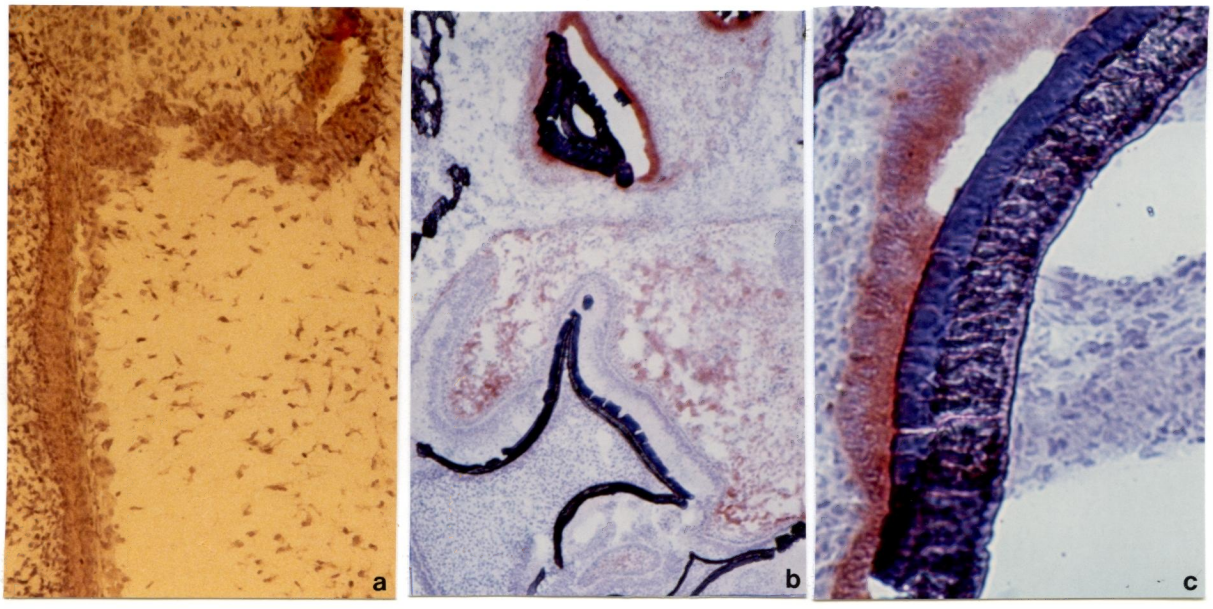
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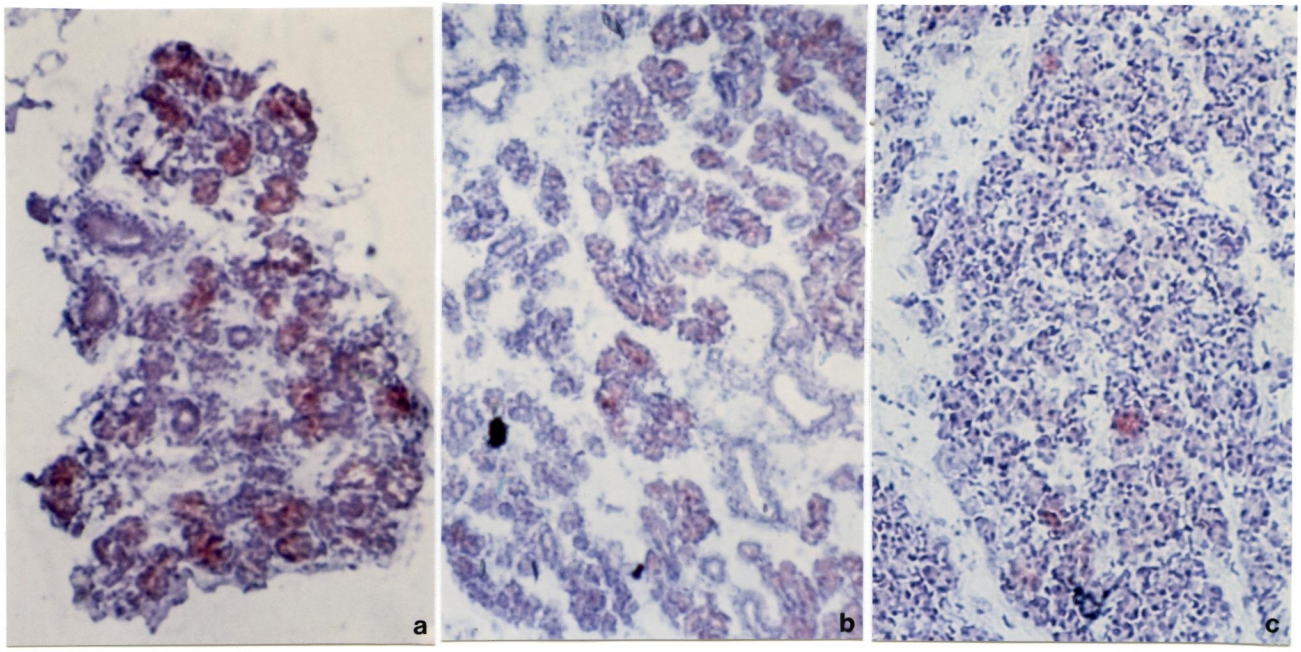
Fig. 3.4. GGT-staining of hamster salivary glands. a) A weak GGT(+) staining of the lobules of acini of minor salivary glands on the section 1 day after birth. b) Positive GGT staining on day 6 after birth. c) GGT(+) staining of parotid gland of adult hamsters (low power view).

Fig. 3.5. GGT-staining on the sections of nasal and sinus mucosa. a) A photomicrograph showing strong GGT staining in the pseudostratified ciliated epithelial lining cells 1 day after birth. b) A moderate GGT staining in similar location on day-3 section after birth (low power view).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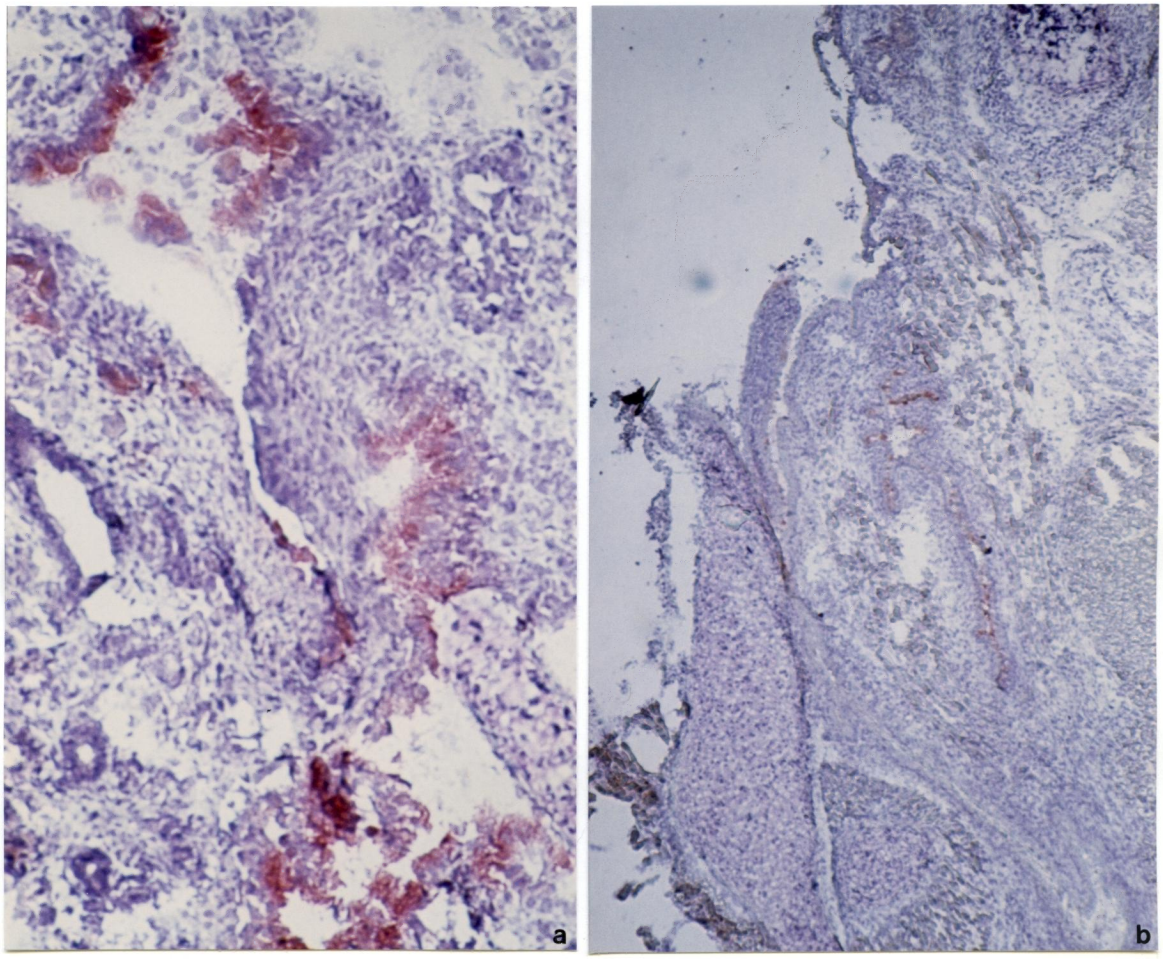
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Fig. 3.6. GGT staining of hamster skin. Photomicrographs showing negative GGT staining in epidermis but strong GGT(+) staining in the germinal matrix of both external and internal hair root sheaths on sections from a) 1 day before birth, b) 1 day after birth, c) 10 day after birth, and d) adult hamsters (low power view).

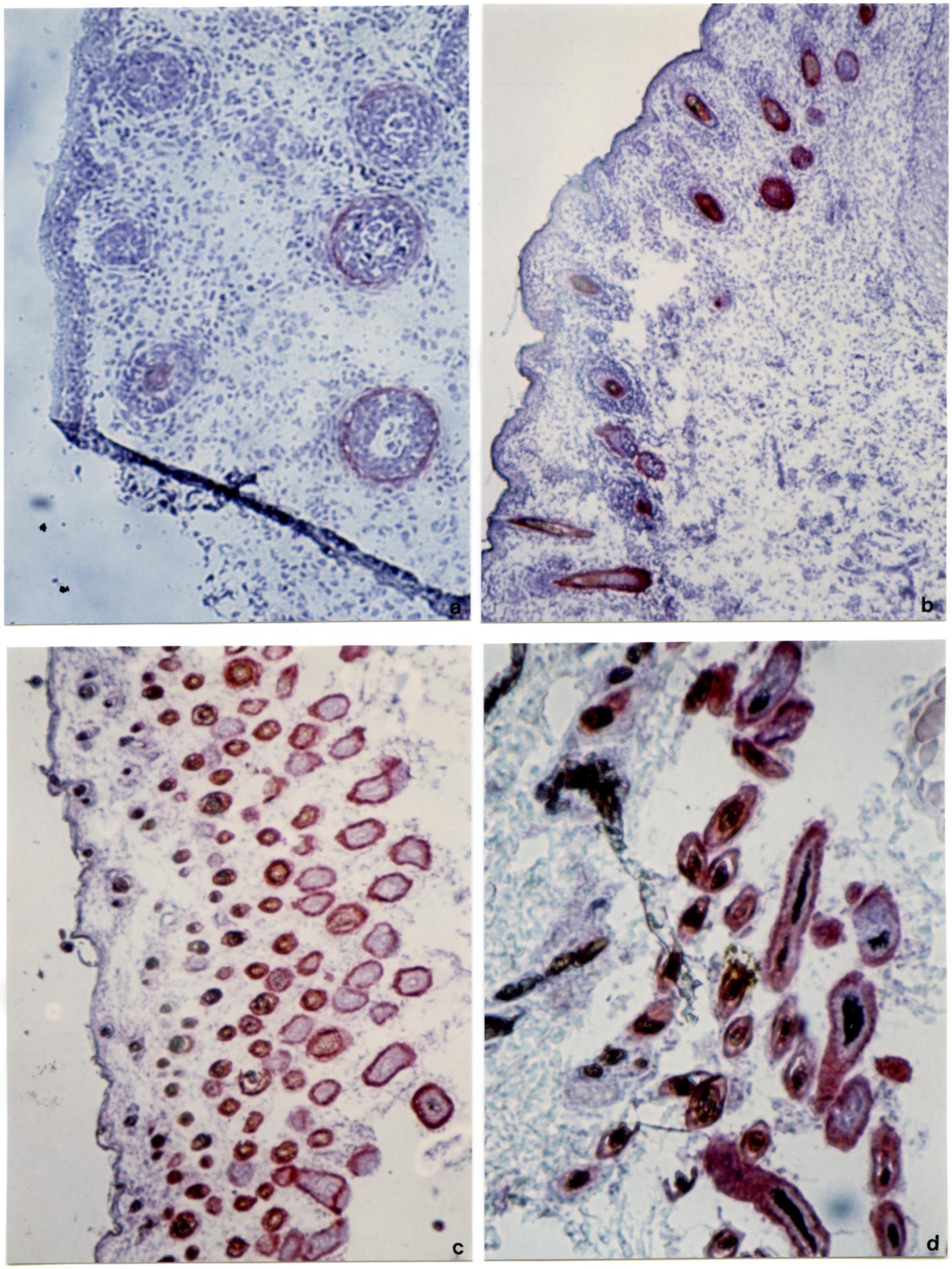
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Fig. 3.7 a-g. GGT staining of hamster kidneys. Photomicrographs showing strong GGT staining in renal tubular cells in all sections from the designed days (low power view).

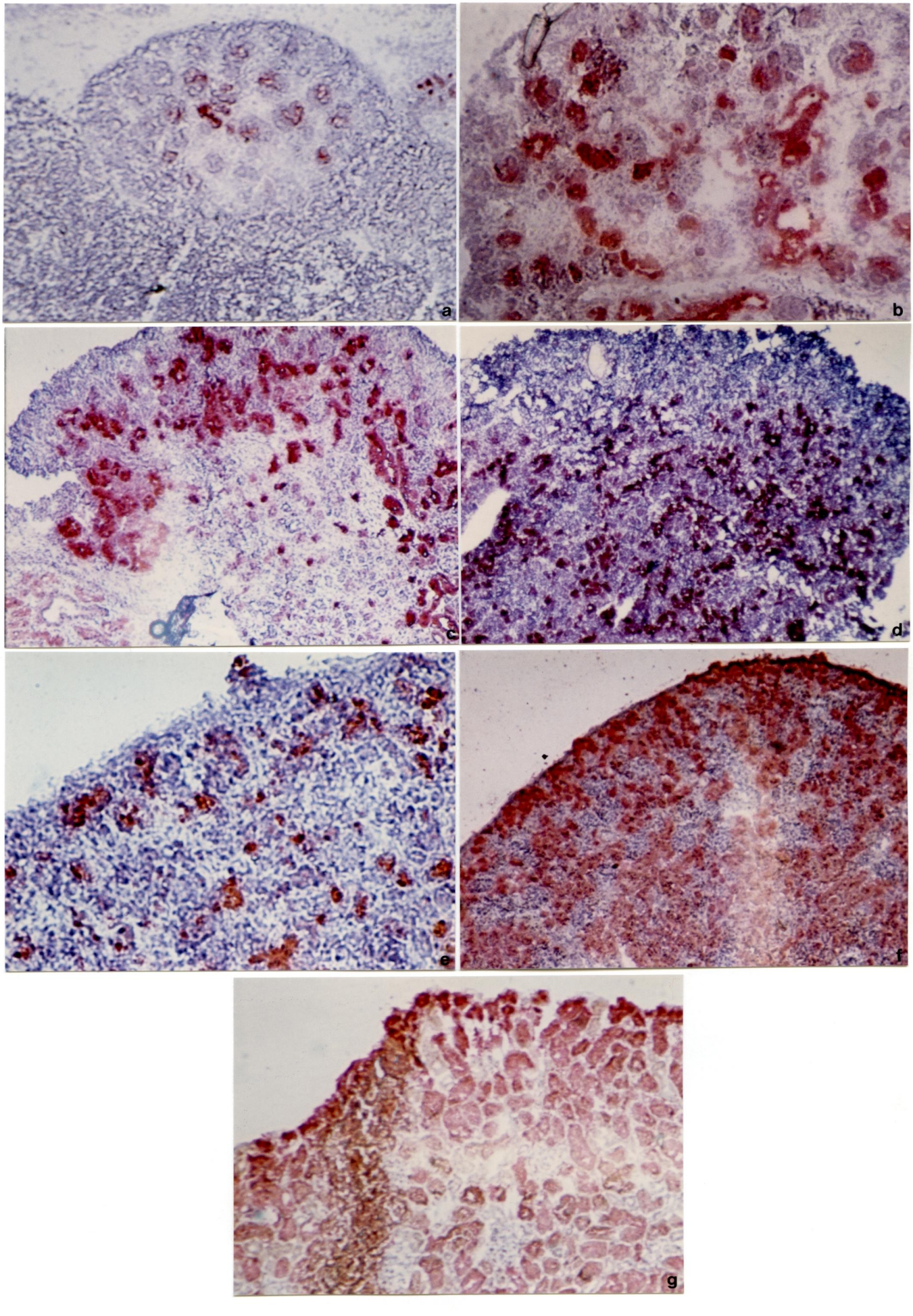
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Fig. 3.8. GST-P antibody staining for hamster livers. a) A diffuse, moderate GST-P staining in hepatocytes 1 day after birth (low power view). b) A weak GST-P staining in hepatocytes 3 days after birth (low power view), c) A negative GST-P staining of hepatocytes 6 days after birth (medium power view) and d) An ambiguous GST-P staining in hepatocytes 10 days after birth (medium power view). e) A moderate GST-P staining in the adult liver hepatocytes (low power view). f) A positive GST-P staining in the control rat liver (low power view).

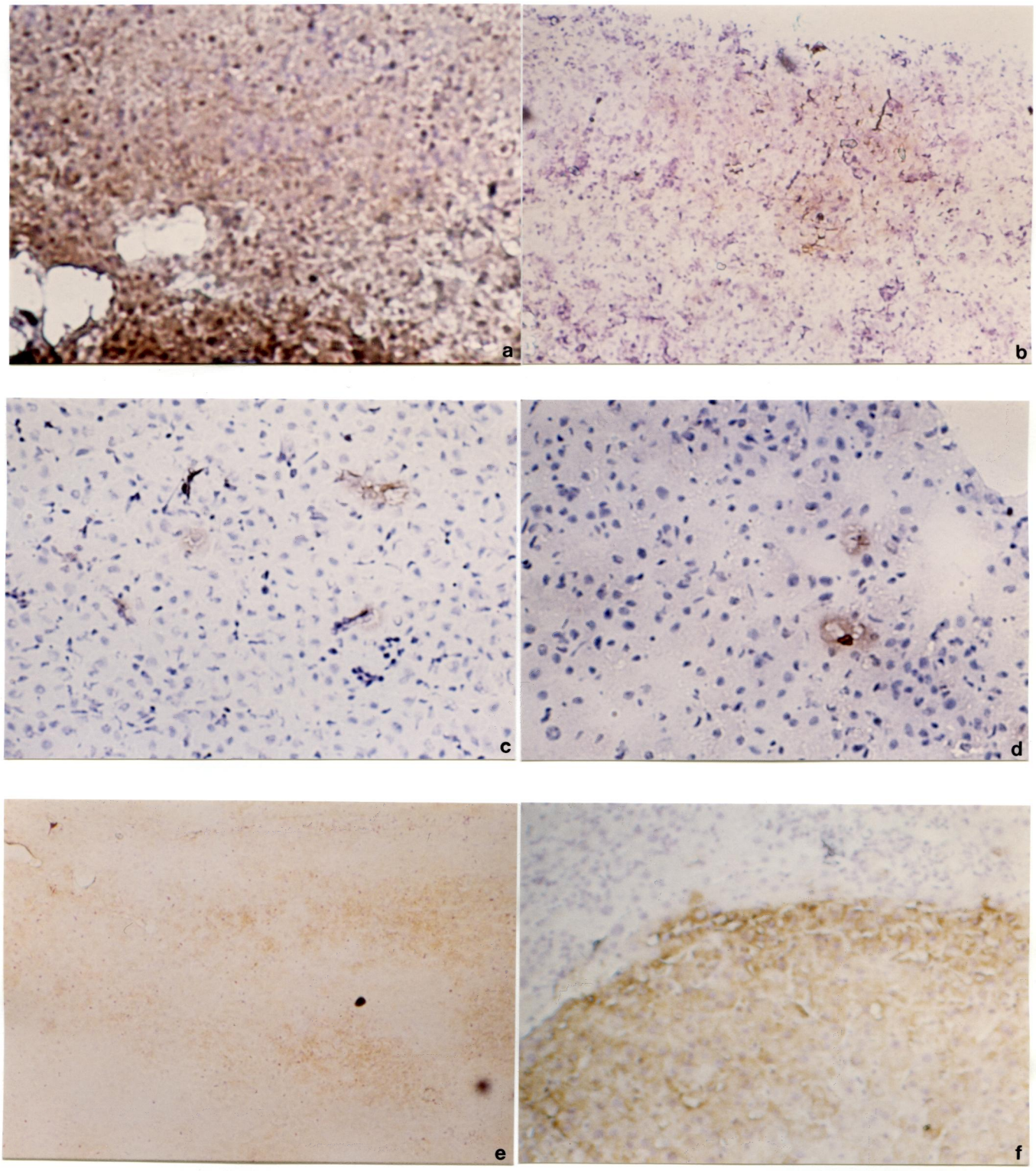
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Fig. 3.9. The enzyme staining of hamster gastrointestinal tracts. a & b) Photomicrographs showing GGT staining in the villi of the lining epithelial cells 1 and 3 days before birth. c) A moderate GGT staining in the gastric glands of adult fundus. d) A weak GST-P staining in the luminal surface of adult stomach lining epithelium. e-i) A strong GGT staining in lining epithelium, particularly the lumical surface of intestine (low power view).

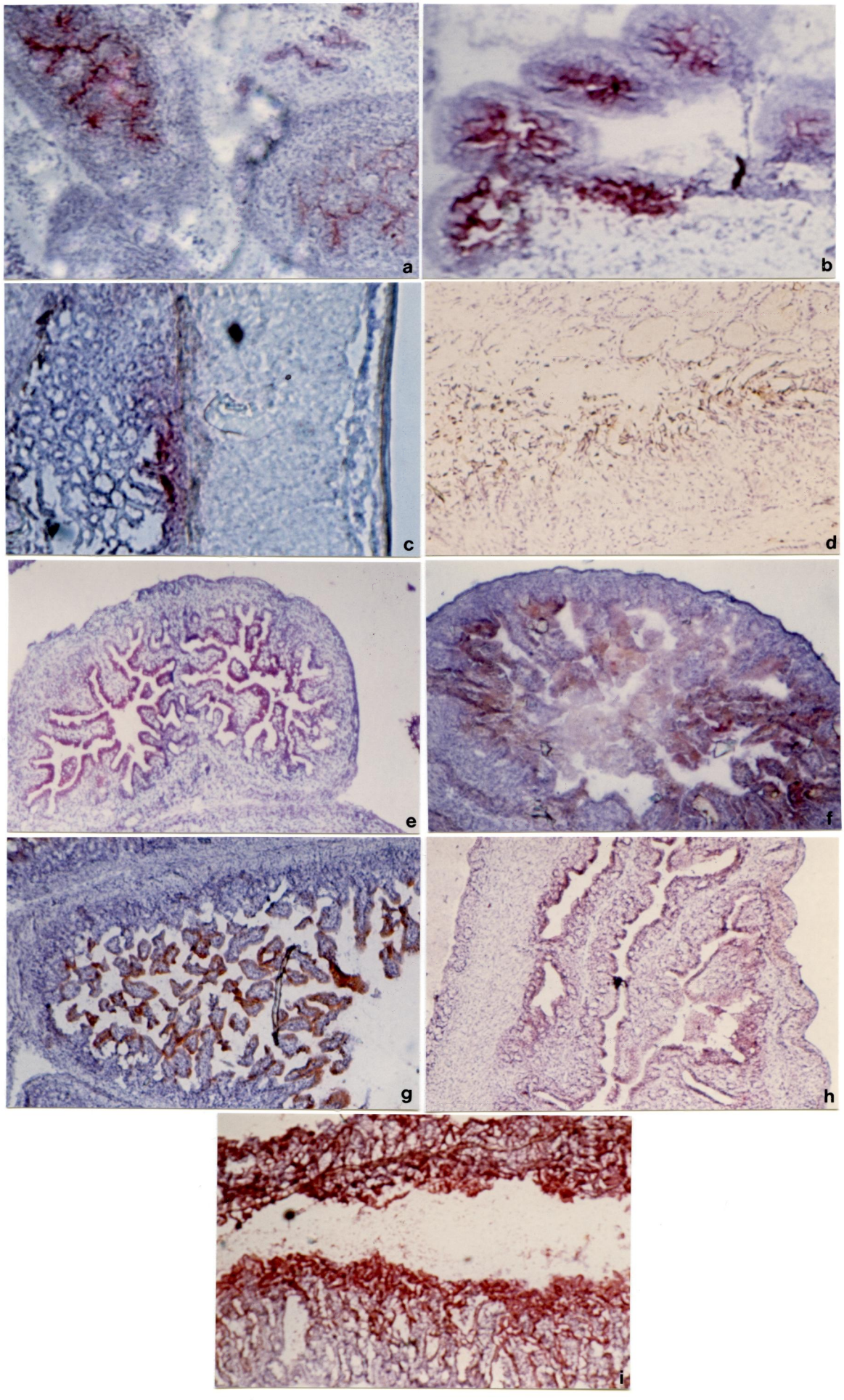
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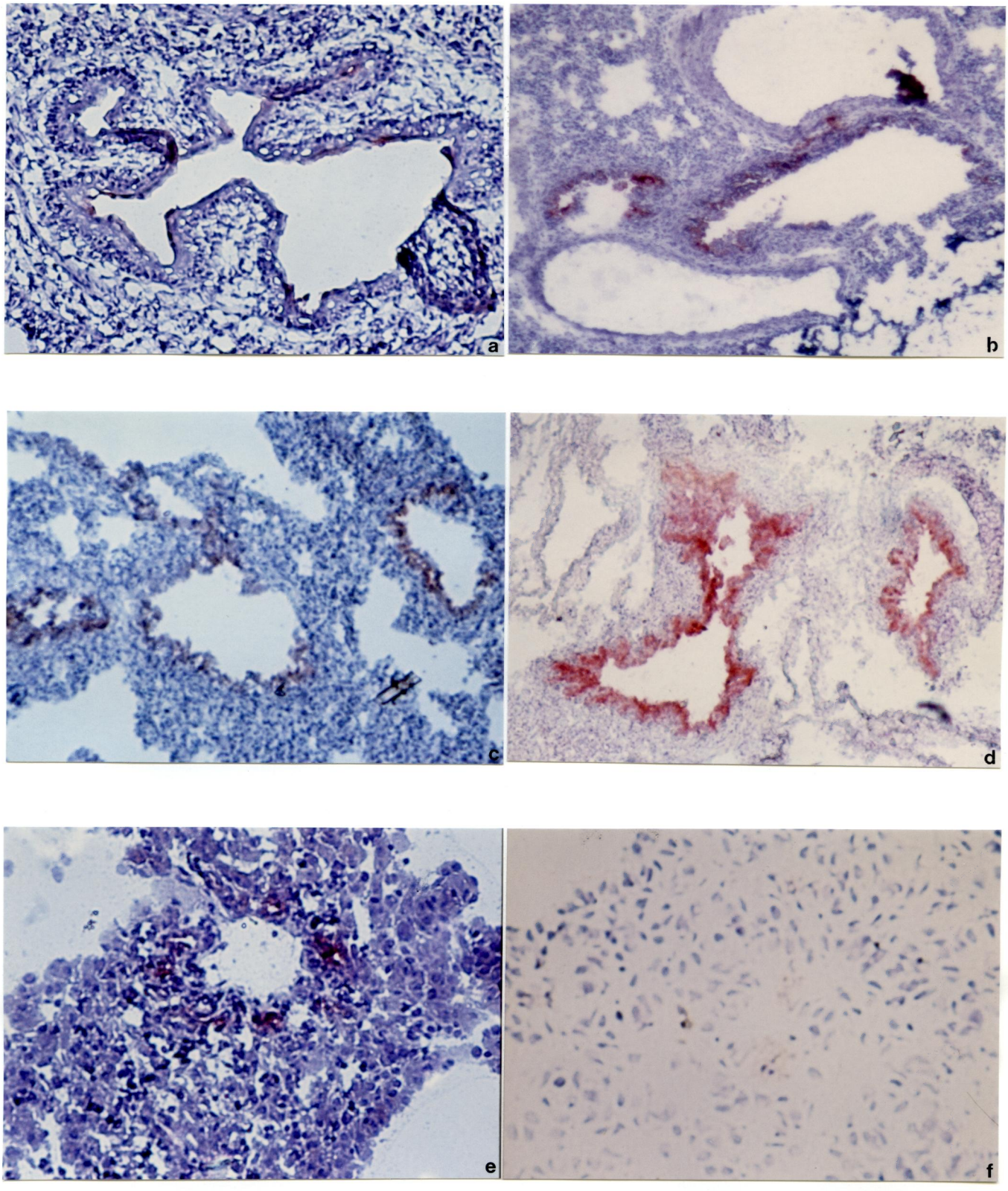
Fig. 3.10. Enzyme staining of hamster respiratory lining epithelium. a-e) Photomicrographs showing GGT staining in the respiratory lining epithelium of trachea, bronchi and bronchioles on 1-, 3-, 6-, 10-day old and adult hamsters respectively (low power view). f) A weak GST-P staining in the similar regions on day 1 sections after birth (medium power view).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