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Pi Class of Glutathione Transferase is the Major Form of Detoxifying Enzyme in the Human Epithelial Tissues and Saliva

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Localization of isoenzyme of glutathione transferase Pi class was compared in different human tissues by immunohistochemical analysis. Strong enrichment of GST-Pi in the epithelial tissues was observed in the granular layer of skin, nipple and esophagus which are vulnerable to exogenous chemicals and in the duct epithelium such as pancreatic, biliary, salivary, renal tubules as well as in the steroid biosynthesis organs such as theca and granulosa of ovary, leydig cell of testis and zona reticularis of adrenal glands. In addition to these immunohistochemical analysis, the presence of GST-Pi enzyme was confirmed in Western blot analysis of human epithelial keratinocyte cell line. However, carcinogenic transformation induced by *K-ras* oncogene transfection in human epithelial keratinocytes did not affect GST-Pi expression. Moreover, the presence of GST-Pi in the human saliva was found by Western blot analysis. These data suggest that GST-Pi enzyme is present in the tissues either vulnerable to environmental toxic substances or producing a lot of endogenous steroid compounds or secreting toxic substances out. Therefore the physiological role of the GST-Pi enzyme is suggested to be one of the major enzymes for detoxification in human tissues.

INTRODUCTION

Glutatione transferase (GST) is one of the major enzymes, responsible for detoxification against the toxic chemicals as a member of phase II reactions (Chasseaud, 1979; Jakoby, 1978). Isoenzymes of GST are recently well characterized and most of the coresponding genes have been cloned and analyzed (Mannervik *et al.*, 1985). Among GST isoenzymes, the acidic form of GST (placental form or GST-π in human and GST-P in rat) has attracted the

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attention of oncologists because the carcinogenic insult would induce GST-P expression very sensitively in the preneoplastic lesion of the murine hepatocarcinogenesis model (Sato *et al.*, 1984; Moore *et al.*, 1985; Satoh *et al.*, 1985). Thereafter, the expression of GST-P has been used widely as the index of the carcinogenic transformation in a variety of animal experiments. However, in applying the concept to human cancer study, the species-difference in the GST isoenzyme pattern of the tissues were observed and the possibility of GST-π as preneoplastic marker in the human tissues has become controversial (Soma *et al.*, 1986; Di Ilio *et al.*, 1987, 1988; Shea *et al.*, 1988; Moscow *et al.*, 1989; Park *et al.*, 1990). Therefore in this experiment, we tried to screen the normal presence of GST-Pi isoenzyme in different human epithelial tissues, prior to analysing its expression in human cancer tissues.

MATERIALS AND METHODS

Reagents

Reduced glutathione, glycine, tris (hydroxy-methyl) aminomethane, nitrobluetetrazolium (NBT), 5-bromo-4-chloro 3-indolylphosphate (BCIP), ammonium persulfate and N, N, N', N'-tetramethylene diamine (TEMED) were purchased from Sigma chemicals (St. Louis, Mo., U.S.A.). Prestained molecular weight markers, acrylamide and N, N'-methylene-bisacrylamide were obtained from Bethesda Research Lab. Inc. (Gaithersburg, U.S.A.) and anti-rabbit IgG-conjugated alkaline phosphatase or protein G-conjugated peroxidase from Boehringer Mannheim Biochemica. The anti GST-π antiobdy (Kwak & Park, 1988) and anti GST-L antibody (Park *et al.*, 1990) previously prepared in our laboratory were used in this study.

In Vitro Culture of Human Epithelia Cells

The immortalized human epithelial keratinocytes derived from foreskin tissues (RHEK-1) and the K-ras gene transfected cell (RHEK-1/k-ras) were kindly donated by Dr. Johng S. Rhim at National Cancer Institute (Bethesda, Md, U.S.A.) (Rhim *et al.*, 1986). The cells were maintained in 10% FBS and Dulbeccos minimal essential medium. The cultured cells were harvested with cell scrapers and were subjected to washing three times with phosphate buffered saline. After washing, the cell pellets were stored in -70° C deep freezer until assay.

Collection of Human Tissues and Saliva

The human tissues were collected at the Department of Pathology, Seoul National University Hospital as the formalin fixed paraffin blocs, which had been subjected to routine histological analysis. And the saliva were collected from the normal healthy adults in the cups with protease inhibitors such as phenylmethyl sulfonyl fluoride and benzamidine. The collected saliva were centrifuged at 30,000 rpm, the supernatants of which were concentrated through lyophilization and stored at deep freeze -70° C until analysis.

Western Blot Analysis

The cultured cells were diluted in HEPES buffer (pH 7.4, 0.1 M) and were subjected to sonication five times for 30 seconds each time with 30 second interval on ice bed. The homogenates were subjected to centrifugation (30,000 rpm for 20 minutes, 4° C), the supernatants of which were used as samples for SDS-PAGE analysis as well as the

concentrated saliva. After SDS-PAGE, the protein fractions on the gels were electrotransferred to nitrocellulose filter papers, which were successively treated with primary antibody to GST-π prepared in our laboratory (1:1000 dilution) and secondary antibody of protein G-conjugated peroxidase (1:2000) dilution), or antirabbit IgG-conjugated alkaline phosphatase. The antigen bands were visualized by peroxidase reactions or alkaline phosphatase reaction of the secondary antibody.

Immunohistochemical Analysis

The formalin fixed-paraffin blocs of the human tissues were subjected to immunohistochemical analysis with successive treatment of primary antibody to GST-π and peroxidase and antiperoxidase complex, followed by visualization with diaminobenzidine peroxidation. For the critical cases, the GST-π enzyme isolated in our laboratory from the human placental tissues was added to the reaction mixture to block the primary antibody in order to exclude the non-specific cross-reactive problem.

RESULTS

1. Isoenzyme Pattern of Glutathione Transferases in Human Epithelial Cell Lines.

The western blot analysis indicated the presence of GST π form in the immortalized human epithelial keratinocyte cell line (RHEK-1) as well as in the K-ras transfected RHEK-1 cell line (RHEK-1/ras), as show in Fig. 1. The transfection of the human epithelial cell with K-ras oncogenes did not cause any increase of GST-π expression in human epithelial cell lines.

2. Immunohistochemical Localization of GST-π in Human Tissues.

Immunohistochemical study showed the presence of GST-π isoenzyme in a variety of human epithelial itssues as summarized in Table 1. The stratified squamous epithelial zones of skin, esophagus, and nipple were strongly enriched with GST-π isoenzymes. The epithelial tissues of bile duct, gall bladder, pancreatic duct, prostate, endometrial gland, renal tubules and bronchial tissue were equipped with GST-π enzyme. Moreover, several tissues, related with steroid metabolism such as zona reticularis of adrenal gland, theca and granulosa of ovary, and leydig cell of testes were observed to have GST-π enzyme. In addition, the central nervous system, peripheral nerve cells and chondrocyte as well as smooth muscle fibers showed positive reaction to anti GST-π antibody.

3. Presence of GST-π Isoenzyme in Human Saliva.

The lyophilized supernatant of saliva was subjected to SDS-PAGE and the successive western blot analysis revealed that GST-π enzyme is present in the human saliva as shown in Fig. 2. However, western blot analysis with anti GST-L antibody did not show the presence of basic GST's in the human saliva.

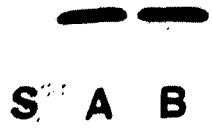

Fig. 1. Western blot analysis of human epithelial keratinocyte cell lines with anti GST-π antibody. (S, prestained molecular weight standards; A, RHEK cell; B, RHEK/ras cell).

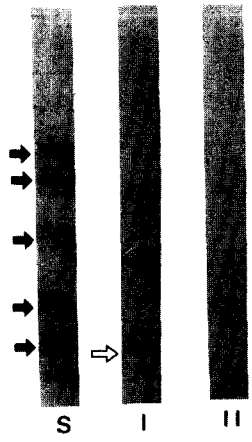

Fig. 2. Western blot analysis of human saliva proteins with anti-GST antibodies. (S, pretained molecular weight standards including β-galastosidase 116 kDa, phosphorylase B 97.4 kDa, bovine serum albumin 66 kDa, egg albumin 45 kDa, carbonic anhydrase 29 kDa; I, Western blot of saliva protein with anti GST-π antibody; II, western blot of saliva protein with anti GST-L antibody. Open arrow indicates 25 kDa GST-π protein).

DISCUSSION

Glutathione transferase conjugates glutathione with a variety of water-insoluble substrates, either endogenous or exogenous, which may detoxify the xenobiotics and lipid-soluble substances via preventing their intracellular acculation and nullifying the toxicity (Jakoby, 1978). Since there are a number of different types of chemicals, unrelated structurally and functionally, it might be expected that there must be as many kinds of glutathione transferases (Mannervik *et al.,* 1985). In fact, many isoenzymes of GST have been reported. Among them, the appearance of GST-P form in the murine preneoplastic lesion of hepatocarcinogenic model suggested that the expression of GST-P might be closely linked with carcinogenic transformation (Sato *et al.,* 1984; Satoh *et al.,* 1985). However, in human study, the expression of GST-Pi (the human corresponding enzyme to GST-P) in the normal liver tissues raised the question on the use of the isoenzyme as a marker

Table 1. Immunohistochemical localization of GST-π in human epithelial tissues.

	Immunoperoxidase staining of glutathione transferase π form
Skin	
epidermis	++
dermis	−
subcutaneous tissue	−
blood vessel	−
sweat gland	+
Nipple	
squamous epithelium	++
sebaceous gland	+
subcutis	−
Breast	
storma	−
myoepithelium	+
duct	+
Esophagus	
squamous epithelium	++
submucosa	±
muscle	±
peripheral nerve	+
Stomach	
chief cell	−
parietal cell	++
foveolar epithelium	+
mucosal muscle	−
nerve	+
serosa	±
Liver	
hepatocyte	+
bile duct	++
Gall bladder	
epithelium	++
submucosa	−
smooth muscle	±
Ileum villous epithelium	±
cryp epithelium	−
lamina propria	−

Continued Table 1.

	Immunoperoxidase staining of glutathione transferase π form
muscularis mucosa	−
smooth muscle	±
nerve	+
Pancreas	
ancinus	−
islet	−
small ductule	+
large duct	+
Lung	
alveoli	+
bronchial epithelium	++
smooth muscle	±
chondrocytes	+
Kidney	
glomerulus	−
proximal tubule	+
distal tubule	++
Bladder	
transitional epithelium	+
submucosa	−
smooth muscle	±
Prostate	
epithelium	+
smooth muscle	±
Testis	
tubule	−
Leydig cell	++
storma	+
Ovary	
stroma	+
granulosa	+
theca cell	+
Adrenal gland	
Zona glomerulus	±
Zona reticularis	+
Zona fasiculata	±
medulla	±

Continued Table 1.

	Immunoperoxidase staining of glutathione transferase π form
Uterus	
endometrial	++
stroma	+
myometrial muscle	−
Uterine Cervix	
endocervical gland	−
endocervical mucosa	−
exocervical squamous epithelium	+
smooth muscle	±
Brain	
cerebrum	+
Placenta	
trophoblast	+
stroma	±
blood vessel	−
Thyroid	
thyrocyte	+
colloid	−

for tumorigenic changes (Park *et al.*, 1990; Soma *et al.*, 1986). Actually in human cancers, the activities of GST were observed to be decreased, contrary to the animal experiment data which showed the higher activities in preneoplastic lesion and tumor tissues. In contrast to the rat hepatoma model, human hepatoma tissue showed the decrease in the expression of basic GST's concomittantly with the increase of GST-Pi expression (Park *et al.*, 1990). Therefore, the neo-appearance or increase of GST-Pi enzyme in the preneoplastic foci or tumor tissues attracted the attention on the differential isoenzyme-specific roles.

The isoenzymes of GST's were classified by the differences in immunological property, substrate specificity and phenotypic expression in the tissues as well as th genetic organization (Mannerik *et al.*, 1985). However, in relation with their physiological functions, the GST isoenzymes are not fully characterized. Moreover, the expression of GST-Pi in the aged cultured primary hepatocytes was observed, in addition to the tumor specific induction of GST-P expression (Kim *et al.*, 1991). Since tumor fissues are more resistent to a variety of toxic chemicals and the aged cultured hepatocytes might be in the state of a stronger endurance to environmenal insults, the expression

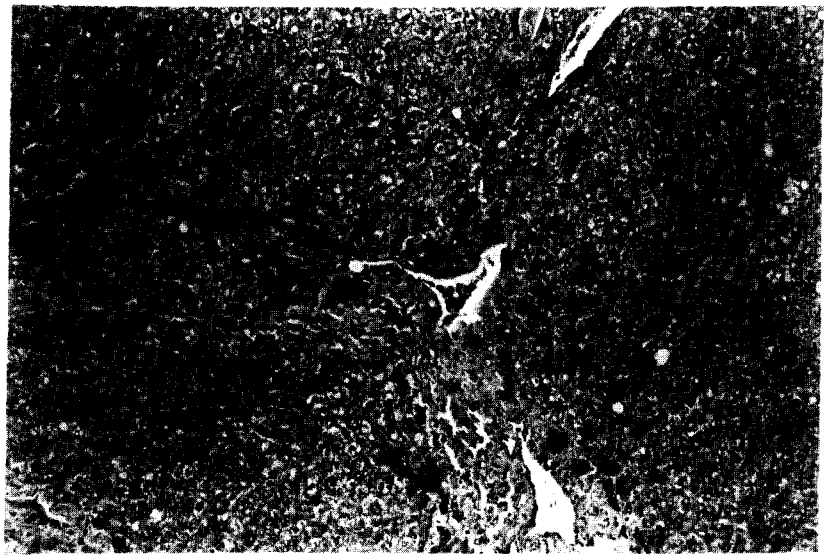

Fig. 3. Immunohistochemical staining of human liver tissues with anti GST-π enzyme is present weakly in normal hepatocytes, while it is strongly enriched in bile duct epithelial cells.

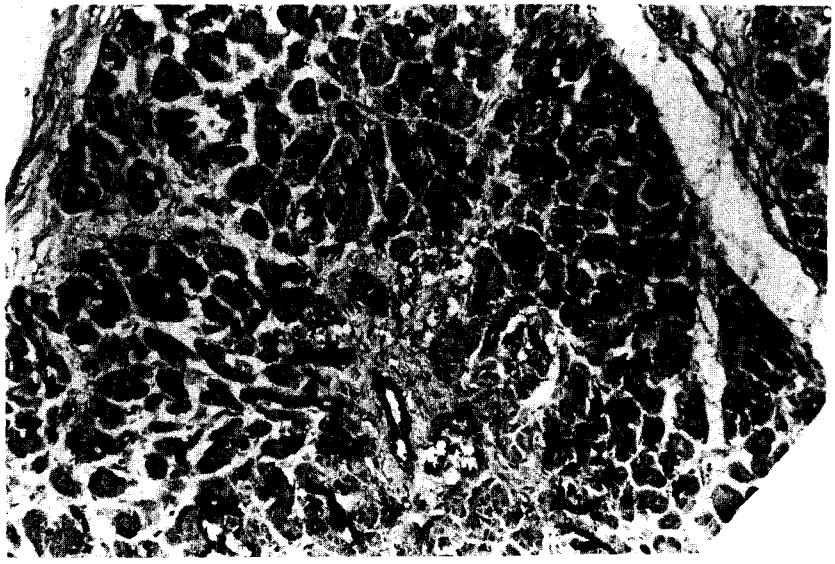

Fig. 4. Immunohistochemical analysis of human pancreas with anti GST-π antibody, which reveals the presence of GST-π enzyme in pancreatic ductule cells.

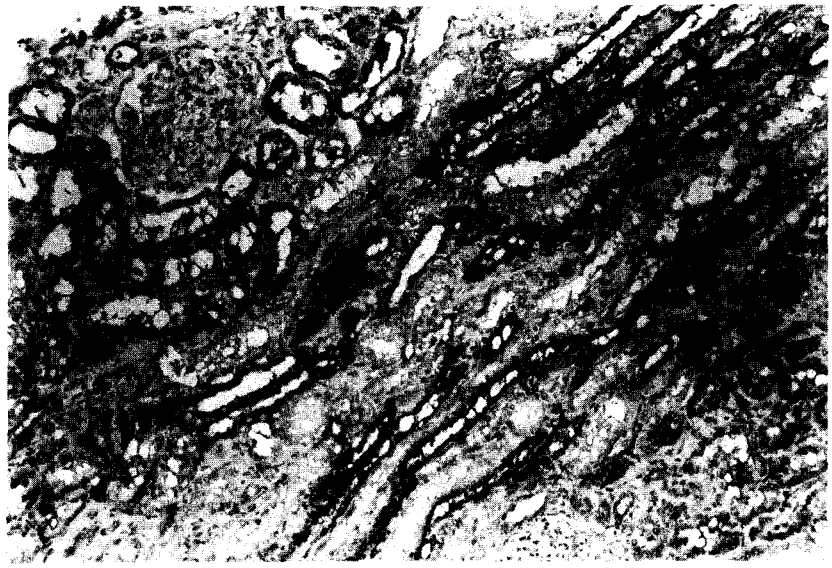

Fig. 5. Immunohistochemical analysis of human kidney with anti GST-π antibody. GST-π enzyme is present weakly in proximal tubular cells, while it is strongly enriched in distal tubule cells.

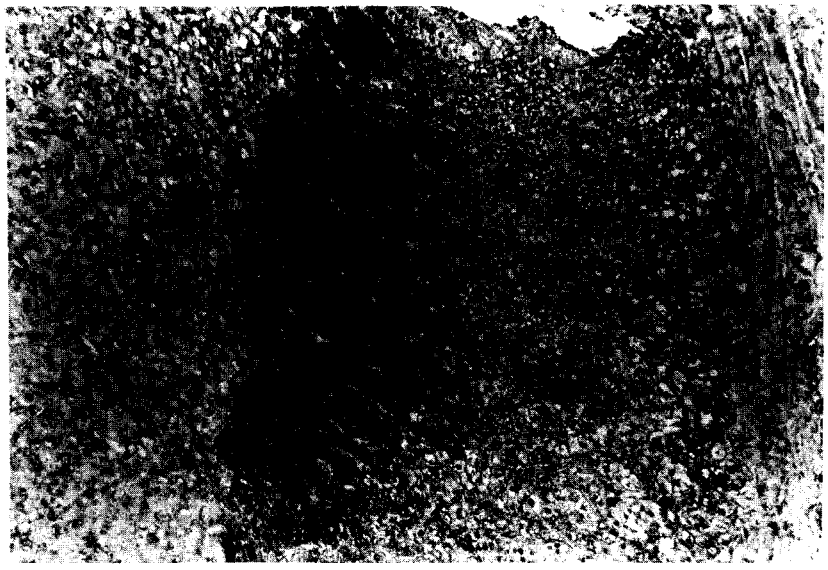

Fig. 6. Immunohistochemical analysis of human adrenal gland with anti GST-π antibody. The GST-π enzyme is strongly localized in the zona reticularis of adrenal cortex.

of GST-P or Pi might be suggested to be the results of cellular adaptation. Therefore, we have proposed in our previous study that GST-P or Pi expression mihgt not be the specific marker for transformation but rather for adaptive response. However, regardless of many reports on the expression of GST-P in the normal and pathological tissues of many species including human, the localization of GST-Pi enzyme was very controversial.

In the present study we tried to screen and compare the expression of GST in the human epithelial tissues. As summarized in Table 1, all the epithelial tissues were strongly positive in the reaction to anti GST-π antibody. All the stratified squamous epithelial tissues from the skin, esophagus, urethra, and nipples were strongly positive. Other epithelial tissues in a variety of ducts such as bile duct, gall bladder, proximal and distal tubules of kidney, large and small pancreatic duct also showed positive reaction to GST-Pi anti-body. In addition, neural tissues and chondrocytes and some steroid biosynthetic organs such as leydig cell of testes, theca and granulosa cell of ovary, and zona reticularis of adrenal gland were reacted positively to anti GST-Pi antibody. These data indicated that the epithelial tissues which are vulnerable to foreign chemicals or toxic environments are well equipped with GST Pi class of enzymes. Moreover, the secretary epithelial tissues responsible for elimination of toxic substances from the body and the steroid biosynthetic tissues are also heavily enriched with GST-Pi enzyme.

The presence of GST-Pi in the epithelial tissues was confirmed by Western blot analysis on human epithelial keratinocyte cell line (RHEK-1). As shown in Fig. 1 the GST-Pi enzyme of 25 kDa size was easily recognized from the supernatants of RHEK-1 cell. Moreover, the transfection of RHEK-1 cell with activated *K-ras* oncogene did not cause any difference in the expression of GST-Pi. This result was contrary to the mouse experiment, where *ras* gene activation would increase GST-P induction. Since the acquisition of tumorigenicity via *K-ras* transfection into RHEK-1 cell was already reported, the activation of *K-ras* was proven (Rhim *et al.*, 1986). Therefore it can be suggested that the human cells are different from murine cells in the relation between GST-P expression and *ras* gene activation, which may explain the species difference in the significance of GST-Pi expression in the preneoplastic lesion or cancer tissues.

In addition to the presence of GST-Pi enzyme in the epithelial tissues, the enzyme was detected in the supernatants of human saliva. As shown in Fig. 2, the western blot analysis confirmed the existence of GST-Pi rather than GST-L in human saliva. Since we used the supernatants of saliva after high speed centrifugation, the enzyme might not be the intracellular cytosolic one, rather it might be secreted from the oral epithelial cells or from the salivary gands. The origin of GST-Pi enzyme in the saliva might require a further study. Since the oral cavity is the major site to entry of all kinds of foods, drugs or whatever into the body, the saliva should be equipped with many of the digestive enzymes and defensive functions. Therefore, the presence of specific isoform of GST-isoenzyme in the saliva itself would imply

its biological importance.

Anyhow, the presence of GST-Pi in the saliva as well as in the epithelial tissues of the human tissues clearly suggests its role as the major component of primary biochemical defense system for the body. It is interesting that the normal human liver tissues, the most active internal detoxifying organ, is enriched with the basic isoenzymes of GST in contrast to the presence of GST-Pi class isoenzymes in the epithelial tissues. Although the significance of the tissue specificity in GST isoenzyme pattern is not clear, the prevalence of GST-P enzyme in cancer itssues would suggest that the expression of GST-Pi might be deeply related with the drug-restance as well as the general detoxifying capability. The low expression of GST-Pi in murine epithelial tissues and its high expression in corresponding human tissue would suggest the evolutionary development in GST-expression (Kwak & Park, 1990).

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인체상피조직 및 타액내 해독효소로서의 glutathione transferase Pi

박상철, 곽상준, 서혜명, 김경옥, 정은미, 최경호, 김우호*

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인체의 여러 조직을 대상으로하여 면역조직화학적방법을 통하여 glutathione transferase isoenzyme의 분포를 비교 분석하였다. 상피조직중의 GST-Pi의 다량분포가 확인되었으며, 특히 외인성 화학물질에 노출되기 쉬운 피부, 유두, 식도 등의 과립층과 췌장, 담도, 타액선, 신세뇨관등의 배설관의 상피 또는 난소의 theca와 granulosa, 고환의 Leydig세포, 부신피질의 zona reticularis 등의 steroid 생합성이 왕성한 부위에서 높은 GST-Pi 분포가 관찰되었다. 면역조직화학적 분석과 병행하여 Western blot 분석을 시행해 본 결과 이러한 GST-Pi 분포가 인체유래 유각상피세포중에서도 확인되었다. 그러나 K-ras 암유전자로 형질전환하여 암화능을 갖춘 인체상피세포주의 GST-Pi 발현은 유의한 차이를 보이지 않았다. 뿐만 아니라 타액중에서도 GST-Pi가 다량 존재함이 Western blot 분석에 의하여 구명되었다. 이러한 결과는 GST-Pi가 인체조직중 외인성독성물질에 노출되는 부위, 내인성 steroid 합성부위 및 배설능을 갖는 부위의 상피조직에 주로 존재하고 있음을 보여주고 있으며, 이러한 GST-Pi의 조직분포는 동효소가 인체의 해독기능에 주요한 역할을 하고 있음을 시사해주고 있다.