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An Immunohistochemical Study of Pi Class Glutathione S-Transferase Expression in Normal Human Tissue

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Glutathione S-transferases (GSTs), a family of isoenzymes that play an important role in protecting cells from cytotoxic and carcinogenic agents, can be separated by biochemical and immunologic characteristics into three distinct classes named alpha, mu, and pi. Previous studies have indicated that there is marked heterogeneity in the expression of different GST isoenzymes in different normal and malignant tissues. To better understand the regulation of the human pi class glutathione S-transferase isoenzyme (GST-π), the tissue distribution of this protein was studied by an immunohistochemical technique using an anti-GST-π polyclonal antibody in normal paraffin-embedded human tissues. These studies indicate that there is a broad distribution of GST-π in normal human tissues and establish a precise localization within the different organs studied. GST-π was expressed predominantly in normal epithelial cells of the urinary, digestive, and respiratory tracts, suggesting a possible role for GST-π in detoxication and elimination of toxic substances. Previous studies have indicated that GST-π and the putative drug efflux pump P-glycoprotein are both overexpressed in multidrug-resistant human breast cancer cells and in xenobiotic resistant preneoplastic rat hyperplastic liver nodules. Results from this study indicate that there are also similarities between the normal tissue distribution GST-π and that previously reported for mammalian P-glycoprotein, particularly in secretory epithelia. This finding suggests that these two gene products, which have been implicated in the development of resistance to cytotoxic drugs, may be coregulated in normal and malignant cells. (Am J Pathol 1990, 137:845–853)

Glutathione S-transferases (GSTs) are a family of isoenzymes that have overlapping substrate specificities and play an important role in the protection of cells from cytotoxic and carcinogenic agents.[1,2] Indeed GSTs are particularly well suited in this regard because these proteins have several properties that can be used to protect cells from toxins.[3] GSTs can conjugate electrophilic substrates, with the tripeptide glutathione resulting in less toxic and more readily excreted metabolites. In addition GSTs can bind and sequester intracellular toxins and certain GST isoenzymes can prevent oxidative damage by an intrinsic organic peroxidase activity that converts toxic peroxides to inactive alcohols. The importance of GST proteins is also suggested by the observations that these proteins are found throughout the animal and plant kingdoms and that GST activity has been detected in almost all human tissues.[4]

Glutathione S-transferase isoenzymes can be characterized on the basis of substrate specificity, sensitivity to inhibitors, isoelectric points, immunologic cross-reactivity, and amino acid composition and sequence.[5] A species-independent classification has been proposed establishing three classes of cytosolic GSTs named alpha, mu, and pi.[5] Nucleotide and amino acid analyses have suggested that the three classes of cytosolic GSTs are the products of separate gene families.[6]

Studies in a rat model of chemical carcinogenesis, the Solt-Farber model, indicate that the pi class GST in the rat (GST-P) is a marker for preneoplastic hepatic lesions as well as in primary carcinogen-induced rat hepatic tumors.[7–10] Although these carcinogen-induced lesions possess many biochemical changes compared to normal hepatocytes, one of the most marked changes in rat liver nodules is an increase in the expression of GST-P. Based on these animal studies, it has been proposed that the pi class GST might be a useful marker for the detection of preneoplastic and neoplastic cells in humans. Indeed increased pi class GST expression has been found in a

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wide variety of human tumors, relative to the normal surrounding tissue, as well as in the serum of patients with cancer.[11-20]

The changes induced by carcinogens in rat preneoplastic liver nodules are also associated with the development of broad spectrum resistance of these altered hepatocytes to hepatotoxins.[21] Similarly multidrug-resistant human breast cancer cells selected for resistance to Adriamycin (Adr^r MCF-7, Adria Laboratories, Dublin, OH) were noted to have a marked increase (more than 45-fold) in the activity of pi class GST.[22] Both model systems of resistance, multidrug-resistant breast cancer cells and rat hyperplastic nodules, are characterized also by overexpression of a 170-kd membrane glycoprotein (P-170) that is believed to function as an energy-dependent drug efflux pump.[23-26] The finding that the pi class GST is increased in both toxin-resistant rat hepatic nodules and in multidrug-resistant human breast cancer cells suggests that the changes in the expression of this isozyme in human tumors may be a marker of drug resistance. Furthermore the finding that P-glycoprotein and pi class GST are overexpressed in these two models of resistance suggests that the regulation of expression of these two genes may be linked and may be related to prior toxin exposure.

To better understand the role of GST-π as a marker of neoplastic transformation or of drug resistance in human tumors, we initiated this study on the characterization of the normal tissue distribution of GST-π protein in human tissues using an immunohistochemical approach. Knowledge of the normal tissue distribution of GST-π should help to define its significance as it relates to the exposure of tissues to xenobiotic challenge and also may provide insights into the factors that regulate the expression of this protein. These studies revealed that there are similarities in the normal tissue distribution of the pi class GST and P-glycoprotein, thus lending additional support to the hypothesis that these two genes may share related regulatory pathways.

Materials and Methods

Immunohistochemistry

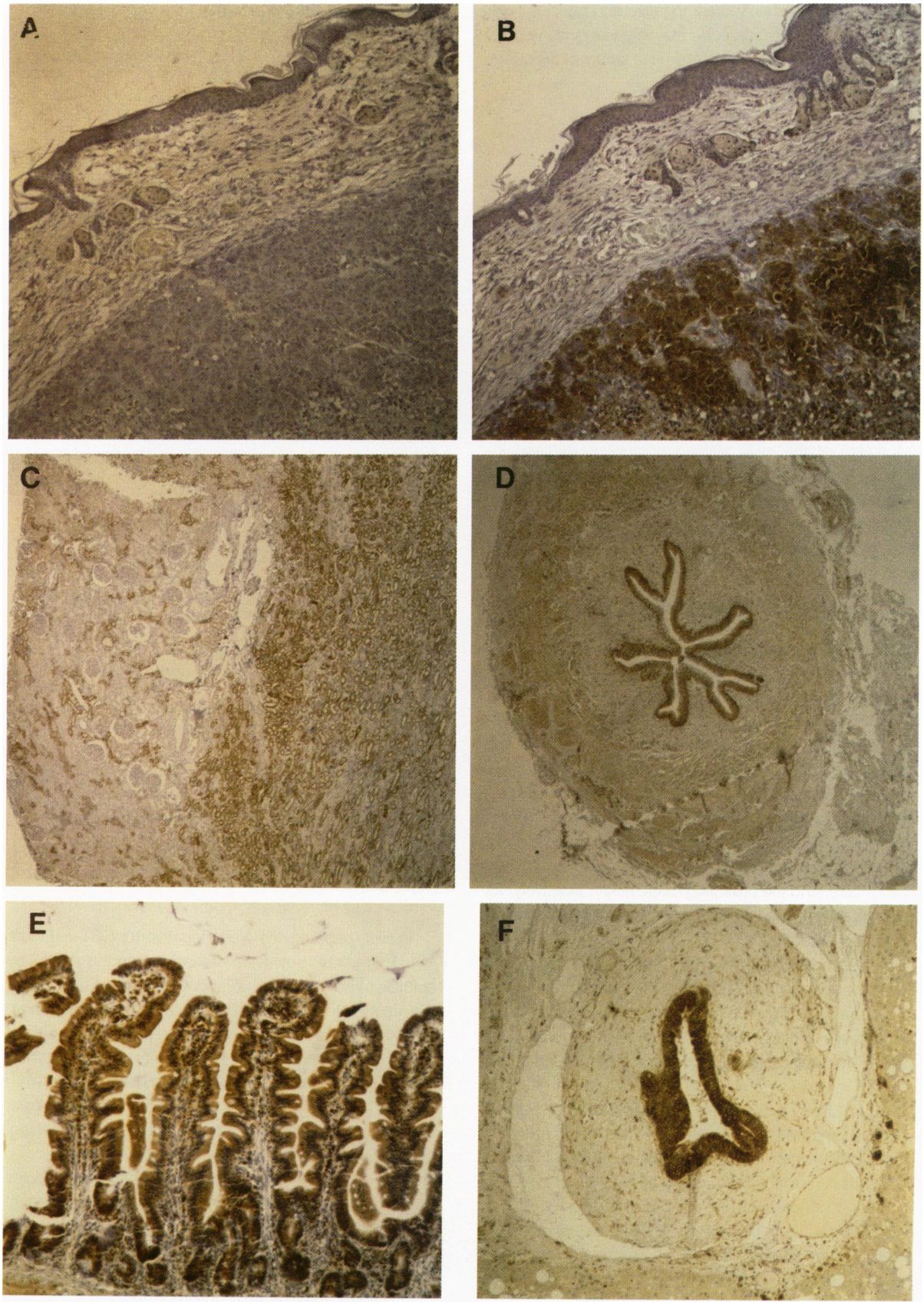
Normal tissues were obtained from the files of the Pathology Departments of the National Cancer Institute, of the Bergonié Foundation (Bordeaux, France), and of the Institute Gustave Roussy (Villejuif, France). Different samples of fresh normal tissues were obtained from surgery and immediately fixed in 10% buffered formalin (BF) or Bouin's solution. File specimens were fixed with one or the other fixative according to the use of each pathology laboratory. The tissues were embedded in paraffin and 5-μm thin sections were cut and laid on gelatin-coated slides.

Antisera against human GST-π was prepared by immunization of 6-week-old New Zealand white rabbits (female) with 100-μg aliquots of purified GST-π at 3-week intervals until a sufficient titer was obtained (at 8 to 12 weeks). Human GST-π was obtained from the Sigma Corporation (St. Louis, MO) and further purified by anion-exchange chromatography on DEAE-cellulose (Whatman, Hillsboro, OR), as described.[27] Purity of the antigen was demonstrated by SDS-polyacrylamide electrophoresis of 2 μg of the final product, which revealed a single band of M^r 23 kd after silver staining of the gel (not shown). Western blotting indicated that the antisera reacted only with human GST-π and not with human alpha or mu class GSTs purified from human liver.[28] Western blot analysis of crude lysates from Adriamycin-resistant human breast cancer cells (Adr^r MCF-7) indicated that this polyclonal antibody reacted with a single 23-kd protein band corresponding to GST-π.[29]

An avidin-biotin complex staining procedure was used (Vector Laboratories Inc., Burlingame, CA). After being dewaxed, slides were rehydrated and incubated with 5% normal goat serum in TRIS-buffered saline (TBS) (50 mmol/l [millimolar] TRIS-HCl pH 7.4, normal saline) as a blocking step to reduce nonspecific staining. Endogenous peroxidase activity was blocked by preincubating slides for 30 minutes with 0.1% H_2O_2 solution in methanol. The polyclonal antisera against purified GST-π prepared in rabbit was diluted 1:400 and applied to the slides for 2 hours. Secondary biotinylated antibody, goat anti-rabbit IgG, was diluted 1:200 and applied for 30 minutes. Primary and secondary antibody dilutions were performed in 5% normal goat serum in TBS. Horseradish peroxidase-conjugated avidin-biotin complex was used according to the directions of the supplier (Vector Laboratories) and incubated for 30 minutes. All incubations were performed at room temperature in moist chamber. Between each step, slides were washed twice in TBS. Immunostains were developed in 0.01% H_2O_2 and 0.05% diaminobenzidine in TBS for 7 minutes at room temperature. Slides were counterstained with hematoxylin, dehydrated, and mounted.

Wild-type (WT) and Adriamycin-resistant (Adr^r) MCF-7 human breast cancer cells[22] were grown as tumors in

Figure 1. *Immunohistochemical detection of GST-π protein in human tumor cell lines and normal human tissues. GST-π protein was examined in cell lines and normal human tissues using an avidin-biotin-complex immunohistochemical technique and hematoxylin conterstaining, as described in Materials and Methods.* **A:** *WT MCF-7 cells grown as tumors in nude mice (×100);* **B:** *Adr^r MCF-7 cells grown as tumors in nude mice (×100);* **C:** *normal human kidney (×25);* **D:** *ureter (×25);* **E:** *jejunum (×100);* **F:** *liver (×100).*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nude mice and were used as negative and positive controls, respectively. Nonspecific staining was evaluated by substituting in the first step preimmune serum of the same animal at the same concentration.

Results

Figures 1A and B depict the results of immunohistochemical analyses of tumors formed in nude mice by WT MCF-7 cells (Figure 1A) and Adr^r MCF-7 cells (Figure 1B). The anti–GST-π polyclonal antibody reacted only with the Adr^r MCF-7 cells. There was no detectable staining of WT MCF-7 cells. This is consistent with Western blot and Northern blot analyses in which no GST-π protein or RNA was detected in WT MCF-7 cells. In contrast, in the Adr^r MCF-7 tumor, which contains high levels of GST-π, the cytoplasm of the cells stained homogenously and intensely. The distribution of the GSTπ protein detected by immunohistochemistry using a panel of different human tissues is shown in Table 1. The GST-π protein reacted with the rabbit anti–GST-π polyclonal antibody in tissue fixed in usual fixatives and paraffin-embedded sections. Staining on surgical samples showed more accurately cellular location than on autopsy organs, where a diffuse pattern mostly was observed.

In the kidney, cortical and medullary tubular structures stained intensely (Figure 1C). This contrasted with the negative staining of the connective tissue in the interstitium and in the capsule. In the glomeruli, the cells of the mesangium and the viceral layer of the Bowman capsule were not stained, although some cells of the parietal layer were faintly positive. The proximal convoluted tubules identified with periodic acid-Schiff stain on serial sections appeared negative. The loop of Henle, the distal convoluted tubule, and the collecting duct stained positively, as did the transitional epithelium of the ureter (Figure 1D) and the urinary bladder.

Digestive epithelium, from esophagus to colon, stained positively for GST-π. In the esophagus, squamous-stratified epithelium stained strongly positive in the basal layers and became less positive in the superficial layers. The surface epithelium in stomach stained faintly for GST-π, while the mucosa in the small and large intestine stained very positive for GST-π in absorptive cells as well as in the mucous-secreting cells. In contrast to the positive staining in the submucosal esophageal glands, there was no staining in the duodenal submucosal glands of Brünner. There was no obvious difference in GST-π expression levels in the lining epithelium of the villi of the small intestine (Figure 1E) and the crypts of Lieberkühn. In gastric glands, parietal and mucous neck cells were faintly positive, while chief cells were negative.

Table 1. *Immunohistochemical Detection of GST in Normal Human Tissue*

Tissue	Main immunoreactive structures
Urinary System	
Kidney	Henle loop, distal convoluted tubule, collecting ducts,
Ureter, urinary bladder	Epithelium, muscularis,
Digestive Tract	
Esophagus	Epithelium, submucosal glands,
Stomach	Surface epithelium and fundic glands,
Duodenum, jejunum, colon	Epithelium, crypts of Lieberkuhn,
Digestive Glands	
Liver	Biliary ducts,
Gall bladder	Epithelium, muscularis,
Pancreas	Ducts,
Respiratory System	
Trachea, bronchi	Epithelium, glands, cartilage,
Lung	Alveolar epithelium,
Reproductive system	
Uterus	Cervical epithelium, Endometrial epithelium and glands, myometrium, Tube epithelium, smooth muscle,
Ovary	Follicles, cortex cells,
Breast	Acinar and ductal epithelium,
Placenta	Cytotrophoblast,
Testis	Seminiferous tubules, epididymis, smooth muscle,
Prostate	Glandular epithelium, smooth muscle,
Endocrine System	
Thyroid	Follicle cells,
Adrenal	Cortex, medulla,
Nervous System	
Brain	Neurones, glial cells,
Peripheral nerves	Schwann cells,
Vascular System	
Heart	Myocardium,
Blood vessels	Endothelium, smooth muscle,
Other	
Skin	Epidermis, sebaceous and sweat glands,
Lymph nodes	Germinal centers.

In liver, hepatocytes were negative for GST-π (Figure 1F). There was, however, intense staining in the biliary epithelium in both the small and large ducts. Furthermore the epithelial lining of the gall bladder was homogeneously positive for GST-π. In pancreas, a pattern similar to liver was noted in that acini and islets were not stained by anti–GST-π antibody but cells lining ducts were strongly reactive. The lining epithelium along the upper and lower airways, the submucous glands, and the alveolar lining cells in the lung also were positive for GST-π.

In the testis, the epithelium of the seminiferous tubules (Figure 2A) was positive, while the endocrine component stained only faintly. The epididymis (Figure 2B) showed strong staining of its epithelium. The basal layer of the prostatic glands was also strongly positive. The epithelia of the vagina, the exocervix, the endocervix, the endome-

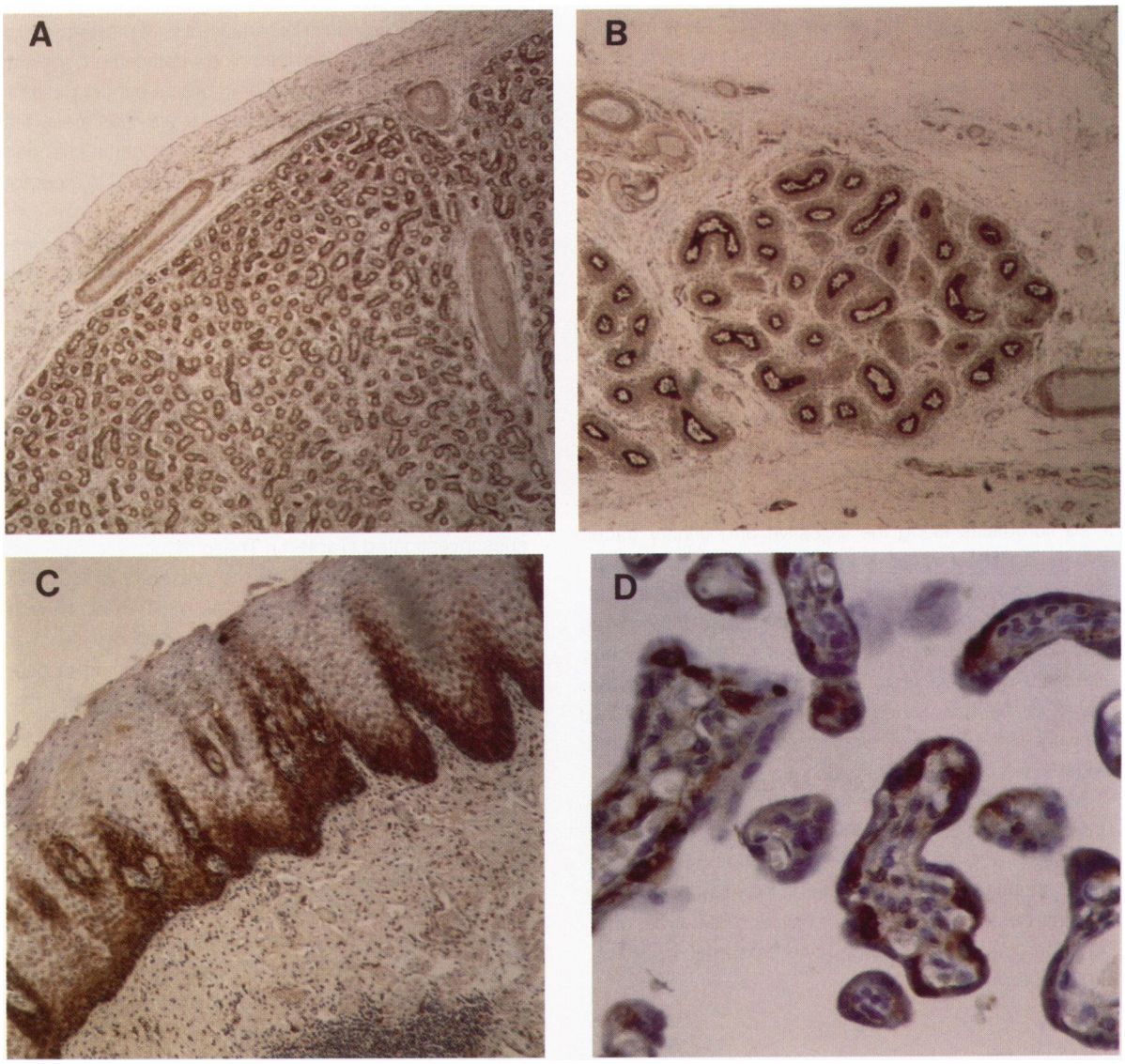

Figure 2. *Immunohistochemical detection of GST-π protein in normal human tissues.* A: *testis, seminiferous tubule (×25);* B: *testis, epididymis (×25);* C: *cervix epithelium (×100);* D: *placenta (×400).*

trium, and the tubes were positive for GST-π. The level of GST-π in the cells lining the exocervix was highest in the basal layers and diminished markedly in the more superficial cell layers (Figure 2C). This was noted previously by Sato and coworkers,[15] who noted increased GST-π staining in patients with cervical cancer and cervical dysplasia. The level of GST-π was higher in squamous cells relative to the glandular epithelium of the cervix. In the ovaries, staining for GST-π varied from intense in the primary follicles to more variable in maturating follicles; follicular cells and granulosa and thecal cells stained faintly.

Breast epithelium also stained strongly for GST-π. There was no obvious difference in the cells of the acini compared to the interlobular ducts. Myoepithelial cells also stained positively for GST-π. Of interest were the findings in human placenta, the organ from which the pi class GST was originally purified. Within the placenta there was marked heterogeneity in GSTπ levels, with cytotrophoblasts strongly positive (Figure 2D).

In the thyroid, staining for GST-π was found in follicular cells but not in colloid. In adrenal glands both the cortex and medulla stained very strongly; in adrenal glands a variable staining of the nuclei was observed. In the skin, the epidermis reactivity with GST-π antibody was very strong in the stratum basalis and decreased in the superficial layers, which is similar to the results in the cervix. In the dermis, sebaceous and sweat glands also stained strongly. In most of the peripheral nerves, Schwann cells were positively stained for GST-π, while the axons were negative. In the brain, only the nuclei of neuronal cells

were positive. In gray and white matter, GST-π was found in glial cells in which both cytoplasm and nuclei stained intensively.

Striated muscle was negative for GST-π. Smooth muscle stained positively in the different organs investigated (vessels, muscularis layer of the digestive tract, gall bladder, uterus, tubes, epididymis, and prostate) but the staining was distinctly fainter than that of epithelial cells. Heart muscle was strongly positive but epi- and pericardium were negative. Chondrocytes (trachea) were positive but the cartilaginous matrix was negative.

Connective tissue stained mostly negative. In particular, the perimysium and endomysium, endo- and pericardium, pleura, thick capsule of organs, loose connective tissue in thyroid, breast, placenta, kidney, and testis were negative. Nevertheless within different organs many cells in connective tissue appeared positively stained. Scattered fibroblasts along the digestive and urinary tracts showed a distinct reactivity in their cytoplasm and/or nuclei. The strongest staining was found in the fibroblasts of the endometrium and in the ovarian cortex. Endothelial cells were positive for GST-π in the capillaries and arterioles of the digestive, respiratory, and genital tracts, in the meninges, the brain, and the skeletal muscle. Cells lining the walls of the lymphatics of the intestinal villi and in the placenta were not stained for GST-π.

Most small lymphocytes present in the mucosa were strongly positive for GST-π. In the follicles in the digestive, respiratory, or genital tract and in lymph nodes, positive small lymphocytes were scattered in the mantle zone and germinal center included larger positive cells corresponding apparently to large lymphocyte cells, macrophages, and connective cells.

Discussion

To understand the regulation of expression of the pi class isozyme of GST, we performed a detailed study using an immunohistochemical technique to examine the distribution of this isozyme in normal human tissues. Previous studies provided evidence for the presence of GST-π protein in various normal human tissue using biochemical methods. In these assays, GST activity was evaluated in the cytosolic fractions of various tissue extracts.[30-33] Unfortunately precise cellular localization of enzymes among different cell types in any tissue sample is not possible by this approach. The use of noncross-reacting antibodies allows a precise immunologic characterization of the different isoenzyme groups[13,16,33] and can be used to perform immunohistochemical detection.[9,12,16,34] In this manner, the cellular distribution of GST-π in normal tissue and tumors can be established. Immunohistochemical methods should allow a more detailed analysis of the expression of GST-π protein in larger samples of patients, which ultimately are needed to determine whether this gene may be a useful prognostic marker in breast cancer patients. This possibility was suggested by a previous study that indicated that GSTπ RNA levels were higher in estrogen-receptor–negative breast cancers compared to receptor-positive tumors.[35]

As previously reported,[8,11] the detection of GST-π was possible using a standard immunohistochemistry technique on paraffin-embedded samples. Immunoreactivity of the antigen was not lost after tissue fixation and paraffin embedding. Such preservation by fixatives used in routine work, such as 10% buffered formaline or Bouin's solution, allow retrospective studies to determine the prognostic importance of GST-π protein.

In the present study, a wide distribution of the GST-π protein was apparent in the panel of normal human tissues investigated (Table 1). These results correlate with previous results establishing the presence of GST-π in many animal tissues.[4] In general, GST-π levels were higher in epithelia, although strong staining also was found in mesenchymal tissues. Similar to what has been demonstrated in the rat,[36] there was heterogeneity of expression of GST-π among different tissues and within the same tissue. As expected, GST-π protein was found mainly in cell cytoplasm. Nevertheless in many types of cells, including epithelial cells and stromal cells, the nuclei also stained intensely. While various histologic procedures may influence immunohistochemical localization, detection of GST-π protein in nuclei also has been described previously in dysplastic epithelium of the cervix.[15] Any relationship between the nuclear location of GST-π and its function remains to be clarified. Recent studies have revealed changes in the intracellular location of glutathione and glutathione-dependent enzymes in HL60 cells selected for resistance to Adriamycin.[37] The significance of this change in intracellular location of glutathione-dependent enzymes and what regulates these changes are not known.

A striking feature of this study was the strong expression of GST-π in the epithelia of the urinary, respiratory, and digestive systems, three major systems involved in elimination of toxic substances from the human body. This further suggests a possible role for GST-π protein in toxin excretion and metabolism. Among normal tissue, kidney has been designated as the richest source for the rat GST-P isoenzyme[9,36] and GST-π has been detected in normal human kidney by Western blot analysis.[14,16,33] In this study, GST-π protein was detected along the nephron in the loop of Henle, the distal convoluted tubule, the collecting duct, and further along the urinary tract in the ureter and the urinary bladder. The presence of the GST-π protein in these nephron structures suggests a possible

Table 2. *Comparison of GST-π and P-170 Expression Detected in Normal Human Tissues by Immunohistochemical Techniques*

	GST-π	P-170*
Kidney	+	+
Ureter, urinary bladder	+	
Esophagus	+	−†
Stomach	+	−
Duodenum, jejunum	+	+
Colon	+	+
Liver		
Hepatocyte	−	+
Biliary ducts	+	+
Gall bladder	+	−†
Pancreas		
Acini	−	−+¶
Islets	−	−
Ducts	+	+
Lung	+	−
Trachea, bronchi	+	+¶
Cervix	+	−
Endometrium	+	−+¶
Tubes	+	−
Ovary	+	−
Breast	+	−†+¶
Placenta	+	−+†+¶
Testis		
Sem. tubules	+	
Epididymis	+	
Prostate	+	−†+¶
Thyroid	+	−†+¶
Adrenal		
Cortex	+	+
Medulla	+	+
Skin		
Epidermis	+	−
Sebaceous and sweat gland	+	+¶
Brain	+	−
Peripheral nerves	+	
Skeletal muscle	−	−†
Smooth muscle	+	
Heart muscle	+	−†
Cartilage	+	
Lymph nodes	+	−†
Spleen	ND	−†

ND, not determined.
* F. Thiebaut et al: Proc Natl Acad Sci USA 1987, 84:7735–7738.
† I. Sugawara et al: Cancer Res 1988, 48:1926–1929.
¶ C. Cordon-Cardo et al: Proc Natl Acad Sci USA 1989, 86:695–698.
In case of discrepancy, results from different studies are mentioned.

role for GSTπ in the elimination of toxic substances in the urine.

Glutathione S-transferase π is also the predominant isoenzyme in normal lung[17] and could take part in the protection of the respiratory tract against volatile air pollutants. Location of GST-π along the upper and lower respiratory tract and in alveolar structures corroborated and extended results previously obtained using immunofluorescence technique.[34] In the digestive tract, high levels of GST-π were found in the small and large intestine. The presence of high levels of glutathione and glutathione-dependent enzymes in all areas of the human intestinal mucosa have been reported.[30] Expression of GST-π along the urinary and digestive tracts could reflect a possible role of this protein in the elimination of toxic and carcinogenic xenobiotics present in the diet.

As discussed above, increased expression of the pi class GST and mdr (P-glycoprotein) genes have been noted in both multidrug-resistant MCF-7 cells and in toxin-resistant rat hyperplastic hepatic nodules. These observations suggested that these genes may share common regulatory mechanisms. Comparison of the normal distribution of these two proteins (Table 2) suggests that there are tissue-specific factors common to the regulation of both these genes.[38–40] Thus both pi class GST and mdr1 genes are expressed in normal the human adrenal glands, thyroid, placenta, prostate, and uterus, suggesting a possible role for both genes in transport and sequestration of hormones. Although there are similarities in the normal tissue distribution of these two genes products, these are also obvious differences. For example, while both genes are expressed in kidney the mdr1 gene product, P-glycoprotein is located primarily in the proximal tubule, while pi class GST is expressed in other areas of the collecting system. Thus, while these genes may share some common regulatory mechanisms, other mechanisms must be involved in the tissue-specific distribution of these two proteins. The finding of increased pi class GST and mdr gene expression in *ras*-transfected rat liver epithelial cells[41] suggests that these genes may be regulated by cellular oncogenes and that GST-π may be useful in screening for malignant transformation in tumors. In fact, several studies have shown that GST-π protein and RNA are increased in certain types of tumors, relative to the adjacent normal tissue. This analysis of GST pi class isozyme distribution in normal human tissues may provide insights into the regulation of expression of this important detoxification isozyme and ultimately should lead to a better understanding of its role in protecting cells from cytotoxic agents.

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