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Cellular and Subcellular Localization of Gastrointestinal Glutathione Peroxidase in Normal and Malignant Human Intestinal Tissue

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The gastrointestinal glutathione peroxidase (GI-GPx) is believed to prevent absorption of hydroperoxides. GI-GPx is expressed in the intestine together with the other three glutathione peroxidase isoenzymes, raising the question of the physiological role of the different GPx types. We therefore studied the cellular and subcellular distribution of GI-GPx in normal and malignant tissue obtained from patients with colorectal cancer or familial polyposis by immunohistochemistry. In healthy ileum epithelium GI-GPx was preferentially enriched in Paneth cells. In unaffected crypts of colon and rectum, it decreased gradually from the ground to the luminal surface. In crypt ground, GI-GPx was uniformly distributed, whereas in cells at the luminal surface it was concentrated in structures capping the nuclei at the apical pole. In colorectal cancer, GI-GPx expression depended on the stage of malignant transformation. In early stages, GI-GPx was increased and pronouncedly associated with the vesicular structures. In progressed stages of malignancy, structures disintegrated and GI-GPx distribution became more diffuse. These observations support the hypothesis that GI-GPx, apart from being a barrier against hydroperoxide absorption, might be involved in cell growth and differentiation.

Keywords: Gastrointestinal glutathione peroxidase; Hydroperoxides; Subcellular localization; Colon cancer; Immunohistochemistry; Selenoproteins

INTRODUCTION

Gastrointestinal glutathione peroxidase (GI-GPx)[1] is so far the fourth member of the family of glutathione peroxidases.[2] In terms of sequence, it most resembles the classical GPx (cGPx),[3] and presumably displays a similar substrate specificity with preference for organic

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hydroperoxides[1] like linoleic acid hydroperoxide.[4] It was first detected in the intestinal epithelium of rats.[5,6] In humans, it is also found in the liver.[1] This particular expression has led to the hypothesis that GI-GPx may function as a barrier against the absorption of hydroperoxides either derived from the diet or endogenously produced e.g. during drug metabolism or inflammatory bowel diseases.[7]

The failure to detect an uptake of intact hydroperoxides in rats fed with oxidized lipids,[8] if analyzed by hydroperoxide-specific methods, is in line with this hypothesis. Also, in the CaCo-2 cell model which has been established to investigate intestinal lipoprotein synthesis and secretion, a release of linoleic acid hydroperoxide from the apical to the basolateral side of the cellular monolayer could only be observed after the monolayer had been damaged. Prevention of hydroperoxide release correlated with the expression of GI-GPx.[4]

Being a selenoprotein, the expression of GI-GPx depends on the availability of selenium. Selenoproteins are not uniformly supplied with selenium but rather they are synthesized according to a strict hierarchy when selenium becomes limiting.[9] The mRNA of selenoproteins is degraded in selenium deficiency. The mRNA of those selenoproteins ranking low in the hierarchy is degraded fastest. GI-GPx is one of the highest ranking selenoproteins investigated so far. This is obvious from the extremely high stability of its mRNA under selenium-deficient conditions, the speed of resynthesis upon selenium repletion, and the difficulty of completely depleting GI-GPx protein by selenium deprivation.[10] Therefore, GI-GPx has been considered the most important of the four types of GPx for peroxide removal in the intestine. However, recent observations led to the hypothesis that it may have additional functions. GI-GPx was detected in estrogen-negative breast tumor cell lines and tissue[6] where it could be induced by retinoic acid treatment.[11] A GI-GPx promoter analysis revealed several GATA sites[12] which have been shown to be implicated in growth and differentiation of intestinal epithelial cells.[13] The GI-GPx gene maps to chromosome14q24.1[14] close to the colon cancer susceptibility locus Ccs1.[15] Finally, a correlation between GI-GPx mRNA levels and resistance against dimethylhydrazine-induced colon cancer in rats was described.[16] These observations do not speak in favour of a simple barrier function.

Here we study the cellular and subcellular distribution of GI-GPx protein in intestinal segments of patients suffering from sporadic colorectal cancer or familial adenomatous polyposis (FAP). We provide immunohistochemical evidence that the localization of GI-GPx differs between the small and the large bowel, as well as between affected and unaffected tissue. This supports the idea that apart from preventing hydroperoxide absorption, GI-GPx might have functions related to cell growth and differentiation, as previously suggested.[6]

MATERIALS AND METHODS

Patients

Tissue specimens from colon, rectum and ileum of patients suffering from sporadic colorectal adenomas or FAP were collected during curative surgical treatment. Morphological intactness of unaffected tissue and the degree of malignancy were verified histologically. At least 10 different sections per patient were analyzed.

Tissue Sampling, Preparation, and Sectioning

Specimens were washed in PBS, and immediately fixed in 5% paraformaldehyde (Merck, Darmstadt, Germany) for at least 24 h. After thorough rinsing with tap water for additional

24 h, samples were dehydrated by graded ethanol and toluol, and then embedded in HISTOPLAST® (Shandon, Pittsburgh, PA, USA) using the full automatic HYPERCENTER®XP (Shandon). Microtome (MICROM, Walldorf, Germany) sections (2 μm) were then mounted on MENZEL Superfrost-slides (Roth, Karlsruhe, Germany).

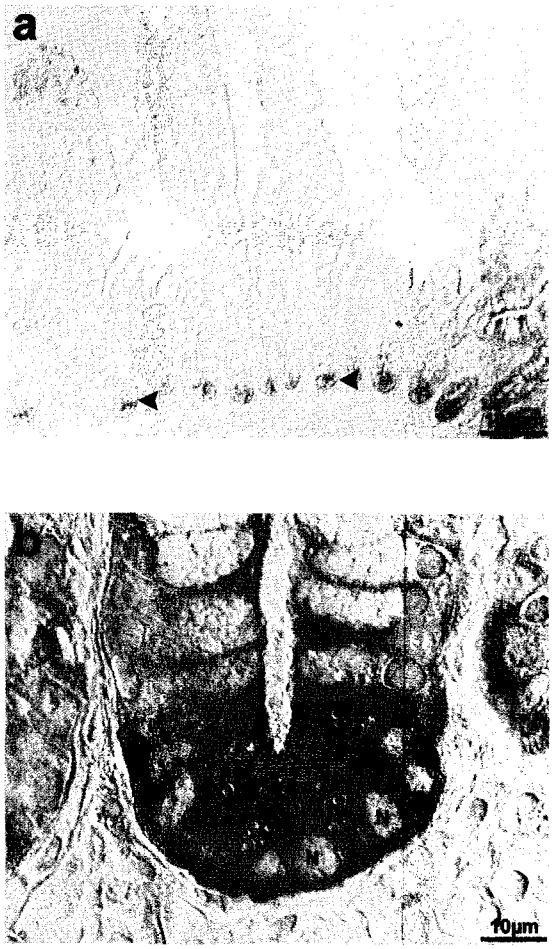

FIGURE 1 Cellular localization of GI-GPx in human ileum. GI-GPx immunoreactivity (IR) (HRP/POD, brown) in healthy ileum from a patient with a sporadic colon tumor. (a) Overview. Enrichment of GI-GPx is visible in crypt grounds within Paneth cells (arrow head). (b) GI-GPx positive Paneth cells containing characteristic granula. Staining is limited to the cytoplasm (arrow head); the nuclei (N) are completely free.

Histology and Immunohistochemistry

The microtome sections were dewaxed in toluol and rehydrated by graded ethanols. For histology, slices were stained with hematoxylin and eosin (HE) (Sigma, St. Louis, MO, USA) and mounted in ENTELLAN® (Merck, Darmstadt, Germany). Antigen unmasking for immunohistochemistry was performed by heat treatment (microwave, 5 min, 850 W) in 10 mM Na-citrate, pH 6.0. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide in deionized water for 10 min. Unspecific binding sites were blocked with 5% goat serum in PBS, 0.2% Triton-X-100 (Sigma), for 45 min, followed by treatment with an Avidin/Biotin Blocking Kit (Vector, Burlingame, CA, USA). A 1:2000 dilution of anti-GI-GPx serum[17] in 1% goat serum in PBS, 0.2% Triton-X-100, was applied overnight at 4°C. Specific antibody binding was visualized by biotin-conjugated sheep anti-rabbit IgG (Dako, Hamburg, Germany), 1:800 in PBS, 30 min at 37°C, followed by a streptavidin–biotin–horseradish peroxidase complex (StreptAB-complex/HRP, Dako) for 30 min and diaminobenzidine (Dako) as substrate according to the manufacturer's directions. Thorough rinsing with PBS, 0.2% Tween 20 (Sigma), was performed after each step. Preimmune serum instead of antiserum served as a negative control. No positive signals were detected under these conditions.

For double labeling, anti-GI-GPx serum (1:800) was diluted together with mouse anti-β-COP (1:150) (clone M3A5, Sigma) and mouse anti-TGN38 (1:300) (Ab-1, Oncogene, Cambridge, MA, USA), respectively. Secondary antibodies, Cy3-conjugated goat anti-rabbit IgG (Sigma) 1:300 and biotinylated goat anti-mouse IgG (Dako) 1:800 were applied one after the other. Biotin was detected by Cy2-labeled streptavidin (KPL, Guildford, UK). Nuclei were stained with HOECHST 33258 (Sigma). Controls included substitution of each conjugate with PBS and

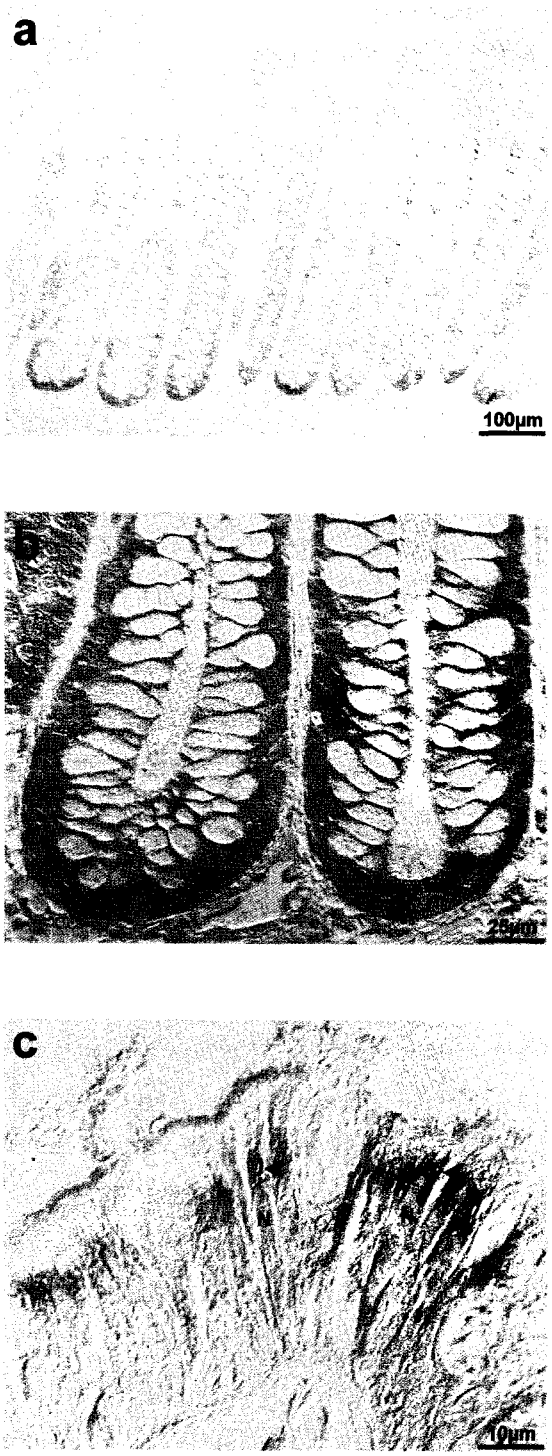

FIGURE 2 Cellular localization of GI-GPx in human colon. GI-GPx IR in unaffected colon from a patient with a sporadic colon tumor. (a) Overview. GI-GPx IR decreases from crypt grounds to apical surfaces. (b) Lower crypt area. (c) Luminal surface of colon crypts. Note the structure-bound concentration of GI-GPx staining apically capping (arrow head) the nuclei (N).

single immunostaining. All steps were done following the protocol described above.

Microscopy and Microphotography

Microscopic investigations were done using the combined light and fluorescence microscope E-1000 (Nikon, Düsseldorf, Germany) with differential interference contrast in combination with a video camera A113C IR-F (Basler, Ahrensburg, Germany) and the True Color Analysis System LUCIA G (Nikon, Düsseldorf, Germany). For confocal microscopy the inverted LSM 410 Laser Scanning microscope (Zeiss, Oberkochen, Germany) was used, equipped with an internal helium–neon laser and an external argon (UV) laser with excitation outputs of 543 and 365 nm, respectively. Images were processed for the color channels red and blue using a 570 nm longpass emission filter for Cy3 detection (red) and 397 nm longpass emission filter for HOECHST (blue) detection.

Prints were made with a Stylus Photo 750 Printer (EPSON, Nagano, Japan) on Photo Quality Glossy Film (EPSON).

RESULTS

GI-GPx Expression in Unaffected Ileum of Patients with Colorectal Cancer

Histologically normal areas of the ileum were analyzed for expression of GI-GPx using the monospecific antiserum raised against the C-terminal peptide from human GI-GPx in rabbits.[17] This serum did not show any cross reactivity with other glutathione peroxidases in Western blots[10] and in immunohistochemistry.[4] Staining of the ileum shows that GI-GPx is only marginally expressed in the epithelium of

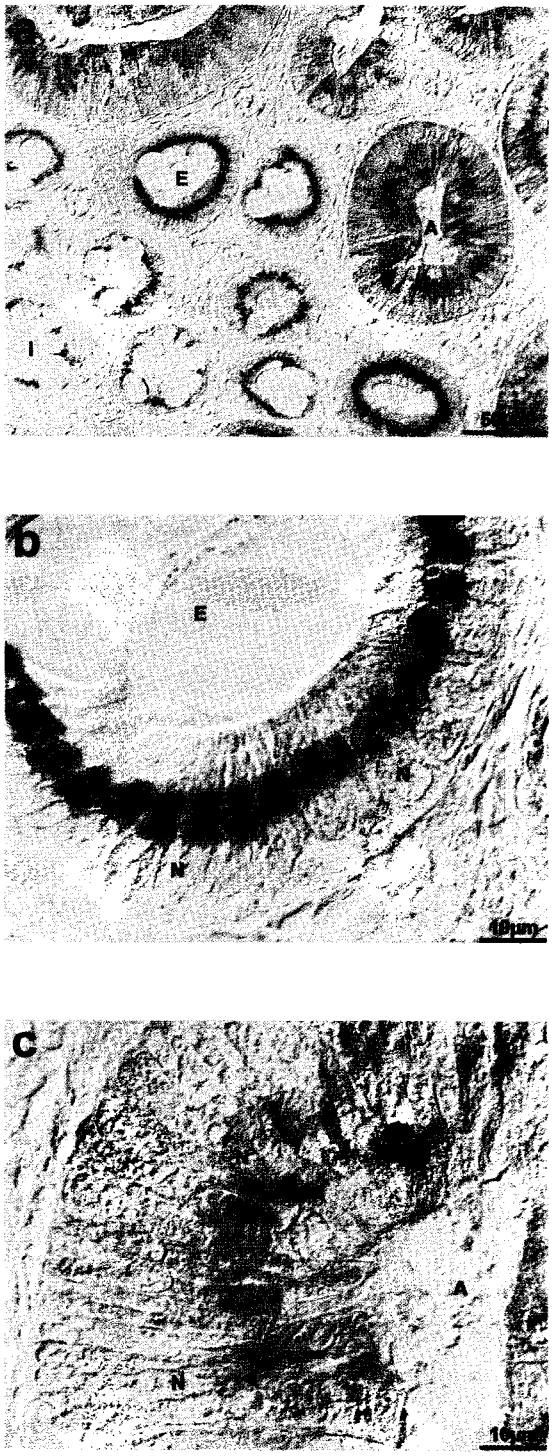

FIGURE 3 Cellular localization of GI-GPx in rectum carcinoma. GI-GPx IR in adenocarcinoma. (a) Overview showing morphologically still inconspicuous crypts (I), crypts showing stages of early transformation (E) and crypts in stages of advanced carcinogenesis (A). (b) In early transformed areas (E) goblet cells are diminished. GI-GPx IR is increased in the epithelial cells with the characteristic localization in nuclear caps (arrow head) again without signals in the nuclei (N). (c) In an advanced stage of carcinogenesis (A) GI-GPx IR becomes more diffuse, less intensive, and distributed throughout the cytoplasm.

the villi, whereas distinct amounts are found in the crypt ground in the cluster of Paneth cells (Fig. 1a). GI-GPx is localized throughout the cytoplasm of the Paneth cells without any positive signals in nuclei (Fig. 1b).

GI-GPx Expression in the Colorectum of Tumor Patients

In histologically intact areas of colon and rectum resected from patients with colorectal cancer, GI-GPx staining is visible in epithelial cells with the intensity decreasing from the ground to the apical part of the crypts (Fig. 2a). In the crypt ground, GI-GPx is distributed throughout the cytoplasm (Fig. 2b). In contrast, staining of the most apical epithelial cells was less uniform. If positive, the cells reveal a specific subcellular localization of GI-GPx in structures capping the nucleus at the luminal side. Goblet cells are apparently devoid of GI-GPx (Fig. 2c).

In adenocarcinoma (Fig. 3a–c), distribution of GI-GPx depends on the stage of malignant transformation. In morphologically inconspicuous crypts directly neighboring the tumor (Fig. 3a, I), it is concentrated in the characteristic structures not only in the luminal epithelial cells but already in the deeper parts of the crypts. In early transformation stages, GI-GPx immunoreactivity is increased and exclusively localized in the typical structure capping the nuclei of the epithelial cells (Fig. 3a, E and Fig. 3b). In advanced stages of malignant transformation (Fig. 3a and c, A), GI-GPx staining becomes weaker, and the structures capping the nucleus disintegrate. Similar observations were made in polyps from FAP patients (not shown).

FIGURE 4 Subcellular localization of GI-GPx. Confocal microscopy of epithelial cells at the luminal surface of morphologically intact colon tissue from a patient with FAP. GI-GPx IR (Cy3, red) (arrow head) is detectable in distinct vesicle-like structures apical from the nuclei (Hoechst 33258, blue) (N).

Subcellular Localization of GI-GPx in the Colon from Patients with Colorectal Cancer or FAP

The GI-GPX immunoreactive structures seen in luminal colon epithelium cells appeared as vesicular structures when investigated by means of confocal microscopy (Fig. 4). Vesicular structures in close vicinity of the nucleus remind of the Golgi system. We therefore tested whether the subcellular localization of GI-GPx in colonic enterocytes might be related to the Golgi apparatus by performing indirect immunofluorescent double labeling using the GI-GPx antiserum and antibodies directed against different Golgi markers. Coatomer vesicles mediate the forward transport from the endoplasmic reticulum to and through the Golgi complex.[18,19] They are mainly located at the cis-Golgi and are coated with coatomer proteins (β-COP). The overlay of GI-GPx with β-COP immunofluorescence revealed a distinct separation of GI-GPx and β-COP (Fig. 5a). β-COP is localized at both the apical and the basolateral side of the nucleus, and its apical localization is between the nucleus and GI-GPx. The trans-Golgi network (TGN), when visualized with TGN38 antibodies, prevails at the basolateral side of the nucleus and, when present at the apical side, does not co-localize with GI-GPx either (Fig. 5b).

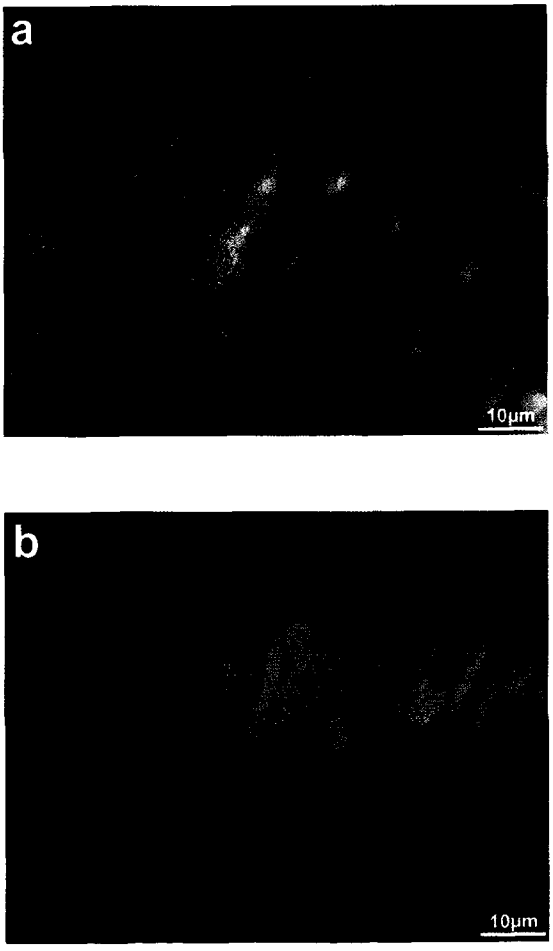

FIGURE 5 Double labeling of GI-GPx with Golgi markers. Superimposed fluorescence and light microscopy of unaffected colon from a patient with sporadic colon carcinoma. (a) Double labeling of GI-GPx (Cy3, red) and β-COP (Cy2, green). (b) Double labeling of GI-GPx (Cy3, red) and TGN38 (Cy2, green). Nuclei were marked with Hoechst (blue). Co-localization of GI-GPx (red) and the Golgi markers β-COP or TGN38 (green) does not occur.

DISCUSSION

(Sub)cellular Distribution of GI-GPx

Investigation of the cellular and subcellular localization of GI-GPx in human intestine led to surprising observations. GI-GPx was not at all uniformly distributed in the epithelial lining of the small intestine and colon. In human ileum it was preferentially expressed in Paneth cells. To connect these findings to the postulated role of GI-GPx as a barrier against hydroperoxide absorption is not trivial. A role of GI-GPx in the Paneth cell-specific metabolism is unclear and, at present, an explanation for the preferential expression of GI-GPx in these particular cells can only remain speculative. Paneth cells are not involved in absorption. They are derived from crypt stem cells and differentiate below the proliferation zone. They produce and release into the lumen a variety of antibiotic proteins like lysozyme, secretory phospholipase A_2 and cryptdins, also called defensins (for review see Ouellette (1997)[20]). The capability to phagocytose and to secrete defensins into the lumen led to the conclusion that Paneth cells contribute to the innate intestinal immunity. Like other phagocytosing and activated immune cells they may produce reactive oxygen species and GI-GPx may protect Paneth cells from being damaged by these radicals. Cytosolic GPx (cGPx) has never been analyzed in Paneth cells either from humans or from any other species. It was, however, enriched in cells derived from crypt grounds from the small intestine of rats.[5] The presence of two types of glutathione peroxidase would guarantee hydroperoxide removal also under selenium limiting conditions for which GI-GPx is predestinated.

In the colon, GI-GPx is organized in distinct structures apically capping the nuclei of luminal epithelial cells. This has never been observed before and is equally difficult to explain in terms of just the postulated barrier function. Vesicular structures close to the nucleus recall the Golgi system. In the small intestine, chylomicrons assembled in the endoplasmic reticulum leave the enterocytes via Golgi-derived vesicles.[21] Thus, the Golgi would be the last cellular site where the release of hydroperoxide-containing chylomicrons into the circulation can be prevented, and the presence of a specific glutathione peroxidase would make sense. However, GI-GPx did not colocalize with generally used Golgi markers. Furthermore, the colon is not the major site of lipid absorption and it remains difficult to explain why GI-GPx should be localized in the Golgi only in the colon. An alternative, tentative interpretation would be that GI-GPx is differently distributed in proliferating, less differentiated crypt ground cells and in differentiated luminal cells prone to be eliminated by apoptosis (see below).

Enhanced GI-GPx Expression during Carcinogenesis

The increased level and the altered intracellular organization of GI-GPx during carcinogenesis indeed suggest a link to malignant transformation.[6] Enhanced GI-GPx complies with the finding of Mörk *et al.*[22] who described an increase in GI-GPx mRNA in human colorectal adenomas, and demonstrates that the enhanced GI-GPx mRNA is indeed translated into protein. The nature of the link to malignant transformation being unknown, potential functions of GI-GPx can only be inferred from what we know about other glutathione peroxidases. cGPx has been shown to be induced by p53 and a p53 responsive element in the cGPx promoter has been identified.[23] An enhanced GPx activity in initiated cells would meet their increased requirement for antioxidant defense thought to prevent tumor promotion.[24] It would counteract further DNA damage by H_2O_2 or other hydroperoxides. The upregulation of cGPx by p53, which itself is activated by DNA-damaging agents, tends to underscore the role of gluta-

thione peroxidases in preventing oxidative DNA damage.[23] On the other hand, cGPx as well as phospholipid hydroperoxide glutathione peroxidase, when upregulated by selenium supplementation or overexpressed, inhibits hydroperoxide-mediated apoptosis.[25–28] Inhibition of apoptosis would prevent the elimination of transformed cells by programmed cell death, an event not necessarily beneficial in malignancy. A role of GI-GPx in the regulation of apoptosis has not been investigated yet. It is however striking that the enzyme disappears from the cytosol and becomes packed into the subcellular structures when apoptosis is required, i.e. in colonocytes at the luminal surface and in transformed cells. In fact, the preliminary histological evaluation of GI-GPx knock-out mice tends to support this view. While the single knock-out mice were asymptomatic, the double knock-outs, GI-GPx (-/-) x cGPx (-/-), displayed a phenotype primarily characterized by shortened villi in the small intestine and increased undifferentiated epithelial cells throughout the gut.[29] This might be a first hint of a role for GI-GPx in the maintenance of normal development and function of intestinal epithelium and complies with the assumption that cGPx and GI-GPx, acting in concert, regulate differentiation and apoptosis during the continuous regeneration of the intestinal epithelium.

The results obtained with GPx knock-out mice do not strengthen the hypothesis that GI-GPx might be pivotal in counteracting oxidative stress in general. Like GI-GPx(-/-) mice, the cGPx (-/-) mice do not show any phenotype. They are, however, more susceptible to oxidative challenge than controls.[30–32] This increased susceptibility is not affected by the selenium status. Selenium-supplemented cGPx (-/-) mice did not react differently from selenium-deficient control mice to oxidative stress. This indicates that cGPx cannot be replaced by any other selenoprotein and makes it unique for the prevention of lethal oxidation.

Taken together, neither the presence in the Paneth cells nor the variation in the expression level and the subcellular localization during differentiation and in dependence on the stage of malignancy is in line with a mere function of GI-GPx as a barrier against hydroperoxide absorption. The observations rather support the idea that glutathione peroxidases have more specialized, even tissue-specific, functions as has impressively been shown for the phospholipid hydroperoxide glutathione peroxidase in the maturation of sperm.[33]

Acknowledgements

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