Virchows Archiv (1994) 424:155–164

ORIGINAL ARTICLE

Terry D. Oberley · Joan M. Sempf
Matthew J. Oberley · Michael L. McCormick
Kenneth E. Muse · Larry W. Oberley

Immunogold analysis of antioxidant enzymes in human renal cell carcinoma

Received: 17 August 1993 / Accepted: 31 December 1993

Abstract Analysis of activities of the antioxidant enzyme manganese superoxide dismutase in human renal cell carcinomas often showed greatly altered enzyme levels (either elevated or depressed) compared to the cell of origin, the kidney proximal tubule. In order to better understand the variability observed, immunogold studies were performed on human renal cell carcinomas using a polyclonal antibody to human kidney manganese superoxide dismutase. For comparison, studies were also performed using antibodies to other antioxidant enzymes. For histologic studies, renal cell carcinomas were subclassified on the basis of light microscopy and ultrastructural analysis into clear cell, granular cell, or mixed clear and granular cell variants. In all three types of tumor, immunogold studies showed little staining using antibodies to copper, zinc superoxide dismutase or glutathione-dependent enzymes. However, intensity of labeling for manganese superoxide dismutase and catalase depended on the cell type(s) in the tumor. Clear cell variants demonstrated trace staining for manganese superoxide dismutase and catalase, while granular cell variants exhibited heavy staining for both of these enzymes. Mixed types of tumors showed clear cells with trace staining for all antioxidant enzymes examined, while granular cells again showed intense labeling for manganese superoxide dismutase and catalase. Using normal kidney proximal tubule as a comparison, immunogold ultrastructural analysis using antibody to manganese superoxide dismutase demonstrated infrequent small lightly labeled mitochondria in clear cell variants, while granular cell variants exhibited numerous medium-sized heavily labeled mitochondria. These data suggest that: 1) the variability in activity values for manganese superoxide dismutase may be due to heterogeneity of cell types in these tumors and 2) manganese superoxide dismutase immunoreactive protein was elevated in granular cells both because of an increase in number of mitochondria and because the labeling density in mitochondria was increased compared to mitochondria in clear cell types or in normal proximal tubular cells.

Key words Manganese superoxide dismutase
Neoplasia · Mitochondria

T. D. Oberley (✉) · J. M. Sempf · M. J. Oberley · K. E. Muse
Pathology and Laboratory Medicine Service,
William S. Middleton Memorial Veterans Hospital, Madison, WI 53705, USA

T. D. Oberley
Department of Pathology, University of Wisconsin Medical School, Madison, WI 53706, USA

M. L. McCormick[1] · L. W. Oberley
Radiation Research Laboratory, University of Iowa College of Medicine, Iowa City, IA 52242, USA

[1] *Present address:* Department of Internal Medicine, University of Iowa College of Medicine, Iowa City, IA 52242, USA

Introduction

Studies of experimental animal tumor models in our laboratories have prompted us to conclude that antioxidant enzymes (AEs) are generally lower in cancer cells than in their normal cell counterparts (Oberley and Oberley 1986). However, data from recent studies have challenged this concept, since at least one AE, manganese superoxide dismutase (MnSOD), has very high activity in certain human tumors, including mesothelioma (Westman and Marklund 1981) and renal cell carcinoma (Yang et al. 1987). In addition, several human tumors show elevated levels of MnSOD immunoreactive protein, including ovarian cancer (Nakata et al. 1992), neuroblastoma (Kawamura et al. 1992), and lung cancer (Iizuka 1984); it is not known whether enzyme activities are correspondingly elevated in these latter tumors, since activity studies were not performed.

The question of whether AEs are altered in cancer is of significant importance since depression of AEs results in elevated levels of reactive oxygen species (ROS), which can result in cell damage (Sies and Cadenas 1985), mutation (Moraes et al. 1991), cell proliferation (Armato

et al. 1984; Shibanuma et al. 1988; Oberley 1985; Burdon and Rice-Evans 1989; Murrell et al. 1990; Craven et al. 1986), and can in fact be carcinogenic (Kennedy et al. 1984). In contrast, recent studies have demonstrated that overexpression of one AE, MnSOD, by transfection of the cDNA for MnSOD suppressed the malignant phenotype of human melanoma cells (Church et al. 1993). Further, recent studies suggest that MnSOD is a tumor suppressor gene in SV-40 transformed human fibroblasts (Bravard et al. 1992a; Bravard et al. 1992b). Elevation of AEs in tumors can have clinical significance since such elevation may cause tumor cells to be resistant to chemotherapeutic agents. It has, in fact, been demonstrated that resistance of certain human tumors to chemotherapy is associated with elevated levels of glutathione transferases (Coles and Ketterer 1990). It has been recently shown in culture that human melanoma cells overexpressing MnSOD are resistant to chemotherapeutic agents known to generate ROS and to irradiation, which is also known to generate ROS (Hirose et al. 1993). Therefore, determination of the level of AEs in human tumors may have practical relevance to human therapy. Most importantly, these combined results suggest that either over- or under-expression of AEs may dramatically affect tumor cell behavior.

Our laboratories previously reported one example of a human renal adenocarcinoma in which MnSOD activity was elevated, although copper, zinc superoxide dismutase (CuZnSOD), catalase (CAT), and glutathione peroxidase (GPX) activities were lower than in the cell of origin, the proximal tubule (Yang et al. 1987). Since a few other studies have now suggested that decreased AEs are not markers of human neoplasia, we decided to examine this issue by studying human renal carcinoma (also called renal adenocarcinoma) in some detail. Our results show heterogeneity in activity and immunoreactive protein in human renal cell carcinomas. Possible implications of these findings for human cancer biology and therapy are discussed.

Materials and methods

Tissue procurement

For biochemical analysis, renal cell carcinomas were obtained from the Cooperative Human Tissue Network in Columbus, OH, USA. Normal human kidney tissue for biochemical analysis was obtained from patient autopsies performed within 24 hours after death. Proximal tubules were isolated from human kidneys as previously described (Yang et al. 1987). All tissue for biochemical analysis was frozen at −70° C until used. Tissues (paraffin blocks) for light microscopic immunogold studies were obtained from the Department of Surgical Pathology at the University of Wisconsin Center for Health Sciences, the combined autopsy facilities of the University of Wisconsin and VA Hospital, Madison, WI, and the Pathology and Laboratory Medicine Service at the VA Hospital. Fresh tumor and normal kidney tissue for immunogold ultrastructural studies was obtained from surgical nephrectomies and immediately fixed for subsequent ultrastructural analysis.

Tissue diagnosis

Light microscopic diagnosis was performed by a team of surgical pathologists at the VA Hospital, Madison, WI and the University of Wisconsin Center for Health Sciences. Multiple sections were made from all areas of each tumor so that they could be correctly classified as clear cell, granular cell, or mixed clear and granular cell types. All tumors used for morphologic analysis in this study had the tumor diagnosis confirmed by electron microscopic analysis by one of the authors [TDO]; however, tumors used for biochemical analysis were not analyzed by light or electron microscopy since the material was obtained from a tissue registry in which pathologic reports but not slides were made available.

Tissue preparation for MnSOD enzyme activity analysis

Sixteen renal cell carcinomas, four normal kidneys, and one sample of isolated human kidney proximal tubules were analyzed for MnSOD enzyme activity. Frozen tissue was thawed and minced in 50 mM phosphate buffer, pH 7.8. Sample preparation consisted of two 15-second bursts with a homogenizer (Tekmar Tissumizer^{R}, Cincinnatti, OH, USA), 50% power, followed by two 15-second bursts with a sonicator (Sonics and Materials, Banbury, CT, USA), 600 watts, 10% power.

Determination of MnSOD activity

MnSOD activity was assessed in whole homogenates by the revised method of Spitz and Oberley (1989).

Protein was determined by the method of Lowry et al. (1951). MnSOD activity was calculated per mg protein. The SOD activity reported here is the average of two determinations on a single sample.

Antibody production and characterization

Polyclonal antisera used included anti-human kidney MnSOD, anti-bovine liver CuZnSOD, anti-bovine liver CAT, anti-human erythrocyte GPX, anti-human glutathione transferase-placenta (GST-P), and anti-rat glutathione transferase-liver (GST-L). The specificity of these antibodies has been previously described and characterized (Oberley et al. 1987; Spitz et al. 1990; Oberley et al. 1990; Oberley et al. 1991b; McCormick et al. 1991; Oberley et al. 1993).

Western blots

Procedures for Western blots have been previously described (Oberley et al. 1987; Spitz et al. 1990; Oberley et al. 1990; Oberley et al. 1991b; McCormick et al. 1991; Oberley et al. 1993). Protein samples were transferred to nitrocellulose from denaturing polyacrylamide gels employing the method of Towbin et al. (1976). The primary antisera dilution for MnSOD was 1:300.

Light microscopy immunogold techniques

For immunogold analysis, 25 renal cell carcinomas were examined: 14 clear cell, 4 granular cell, and 7 mixed clear and granular cell tumors. The tumors were classified on the basis of examination of multiple hematoxylin and eosin stained sections from various areas of each tumor and following ultrastructural analysis. The micrographs presented in the Results are representative of the results obtained with each tumor type. In addition, two normal adult kidneys were used for light microscopic immunogold analysis. Other adult kidney tumors, including papillary carcinoma, chromophobe cell carcinoma, collecting duct carcinoma, and oncocytoma were all excluded from this study. Tumors selected for this study were analyzed using immunogold methods since we

have demonstrated that this method is more sensitive than immunoperoxidase techniques and therfore of more utility when examining routine fixed archival material (unpublished observations).

Four-μm sections were cut from paraffin blocks, mounted on slides coated with 3-aminopropyltriethyloxysilane to ensure adhesion during processing, and dried overnight. The slides were then placed in an oven at 56° C for 60 min to melt the paraffin. Removal of paraffin was accomplished by placing the slides sequentially in xylene four times (10 min each), 100% ethanol two times (2 min each), 95% ethanol, 70% ethanol, 50% ethanol, and distilled water (2 min each). The slides were then washed in Tris-buffered saline (0.05 M Tris, 2.5% NaCl, pH 7.4: TBS). They were incubated with 100 μl of a 1:10 dilution of normal goat serum for 15 min. After removing the serum, 100 μl of rabbit primary antibody (1:200 dilution) was added to the tissue sections, which were then incubated overnight at 4° C. The sections were rinsed in TBS with 0.1% bovine serum albumin for 5 min. The buffer was removed, and then secondary antibody conjugated to 5 nm colloidal gold particles (1:100 dilution) was added for 60 min at room temperature. The sections were rinsed again in TBS with 0.1% bovine serum albumin for 5 min. They were then fixed in 2.5% glutaraldehyde in TBS for 15 min and washed thoroughly in distilled water. Silver enhancer solution was added (Sigma Chemical, St. Louis, MO, USA). The sections were developed in the enhancer until the desired staining intensity was reached (5–10 min). The sections were rinsed in distilled water and placed in 2.5% aqueous sodium thiosulfate for 2 min. They were washed in distilled water, counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene, and coverslipped. Controls contained saline or normal rabbit serum (Sigma Chemical) in place of the primary antibody.

Conventional electron microscopy

Techniques for electron microscopy have been previously described (Gonzalez et al. 1990).

Immunoelectron microscopy

Three cases of clear cell variant of renal adenocarcinoma, three cases of granular cell variant of renal adenocarcinoma, one case composed of mixed clear and granular cells, and two normal kidneys were studied by immunoelectron microscopy. Procedures and controls for immunogold electron microscopy have been previously described (St Clair et al. 1992; Coursin et al. 1992). Distinct cellular and subcellular labeling greater than background was regarded as positive. Cells were then evaluated for relative degree of probe density, ranging from strongly immunoreactive to no label at all. Results presented in this study were from samples treated identically and labeled at the same time and therefore labeling intensity was directly comparable.

Results

MnSOD activity in human renal tissues

MnSOD activity in human renal cell carcinomas showed great variation (7 U/mg–1235 U/mg protein [Table 1]). More importantly, these values were often either elevated or depressed in individual tumors compared to normal kidney tissue (144–152 U/mg protein). Compared to the cell of origin (the proximal tubule = 126 U/mg protein), one renal cell carcinoma had approximately ten times more activity, while another renal cell carcinoma had 18 fold less activity. Compared to normal kidney or proximal tubule, histogram analysis showed 5 tumors with significantly elevated activity, 5 tumors with significantly depressed activity, and 6 tumors with activities similar to control normal kidney (data not shown). Western blot analysis of each of these specimens showed co-migration of bands with purified MnSOD and levels that generally agreed with the activity values (data not shown). It should be emphasized that renal adenocarcinomas used for biochemical analysis were not subclassified into clear cell, granular cell, or mixed cell variants. We decided to further analyze the reason(s) for the variability observed by performing morphologic and immunogold analysis of selected renal cell carcinomas.

Light microscopy

Tumors for histologic analysis were subdivided into clear cell variants, granular cell variants, or mixed tumors based on cytoplasmic staining properties following routine hematoxylin and eosin staining. Light microscopy demonstrated that clear cell variants had clear, light staining cytoplasm, while granular cell variants had intensely dark red staining (eosinophilic) cytoplasm. Mixed tumors were composed of both cell types in varying proportions.

Conventional electron microscopy

All tumors used in this study for histologic analysis were also examined by electron microscopy. Clear cell vari-

Table 1 MnSOD activity in human renal adenocarcinomas and normal renal tissue*

	Sample	Activity (U/mg)		Sample	Activity (U/mg)
# 1	Renal Adenocarcinoma	154	#12	Renal Adenocarcinoma	59
# 2	Renal Adenocarcinoma	714	#13	Renal Adenocarcinoma	180
# 3	Renal Adenocarcinoma	326	#14	Renal Adenocarcinoma	53
# 4	Renal Adenocarcinoma	1,075	#15	Renal Adenocarcinoma	7
# 5	Renal Adenocarcinoma	1,235	#16	Renal Adenocarcinoma	56
# 6	Renal Adenocarcinoma	634	#17	Normal Kidney	151
# 7	Renal Adenocarcinoma	651	#18	Normal Kidney	182
# 8	Renal Adenocarcinoma	241	#19	Normal Kidney	156
# 9	Renal Adenocarcinoma	141	#20	Normal Kidney	144
#10	Renal Adenocarcinoma	25	#21	Isolated Human	126
#11	Renal Adenocarcinoma	217		Kidney Proximal Tubule	

* Results presented as the average of two determinations

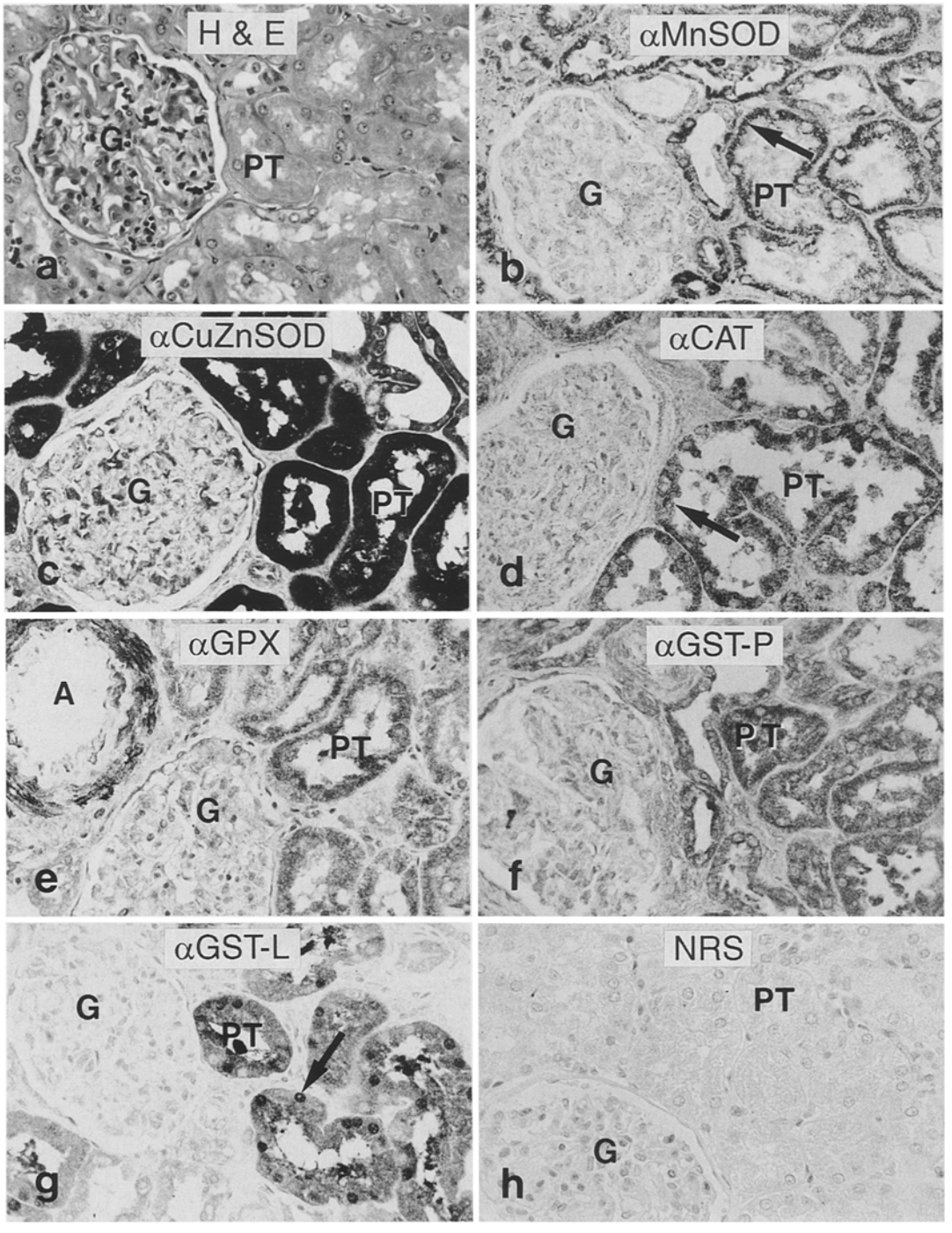

Fig. 1 Immunogold staining of human proximal tubule and glomeruli. (**a**) Hematoxylin and eosin (H and E) stain of proximal tubules (*PT*) and glomeruli (*G*). (**b**) Anti-MnSOD. Proximal tubules demonstrated heavy granular cytoplasmic staining (*arrow*). Glomeruli exhibited trace staining. (**c**) Anti-CuZnSOD. Proximal tubules showed intense nuclear and diffuse cytoplasmic staining. Glomeruli exhibited trace staining. (**d**) Anti-CAT. Proximal tubules demonstrated heavy granular cytoplasmic labeling (*arrow*). Glomeruli exhibited trace labeling. (**e**) Anti-GPX. Proximal tubules showed heavy granular and diffuse cytoplasmic labeling. Glomeruli demonstrated light staining. Moderate staining of smooth muscle cells of renal arteries (A) was observed. (**f**) Anti-GST-P. Proximal tubules exhibited heavy diffuse cytoplasmic labeling. (**g**) Anti-GST-L. Proximal tubules showed heavy nuclear (*arrow*) and diffuse cytoplasmic staining. Glomeruli did not show staining. (**h**) Normal rabbit serum (NRS). Tissues did not show significant staining (**a–h**) × 700

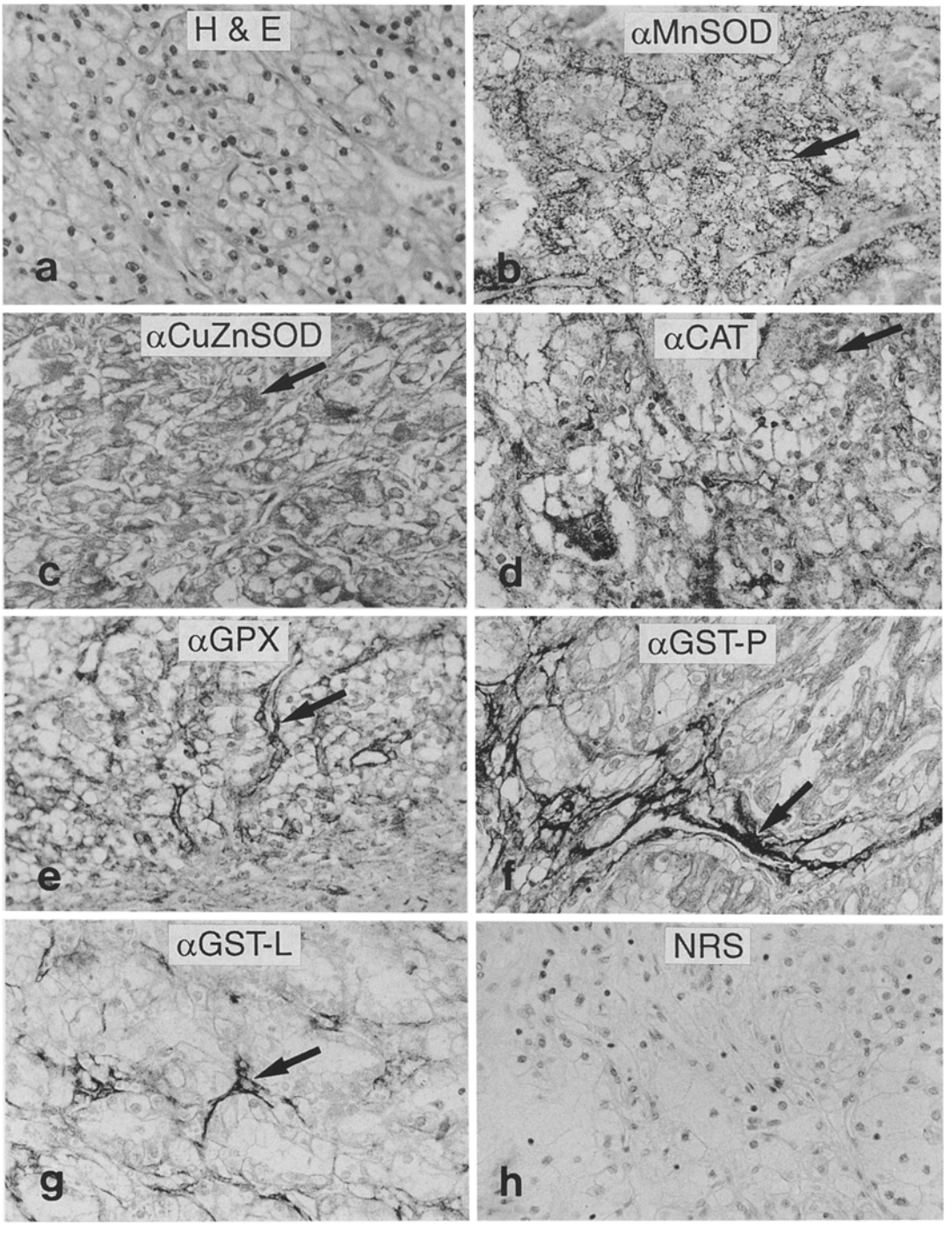

Fig. 2 Immunogold staining of a clear cell variant of human renal cell adenocarcinoma. (**a**) Hematoxylin and eosin (H and E) stain. Tumor cells were arranged in nests or glands. The nucleus was relatively small and the cytoplasm was clear. Tumor cells showed light granular peripheral cytoplasmic staining for: (**b**) Anti-Mn-SOD (*arrow*) and (**d**). Anti-CAT (*arrow*). These cells showed light diffuse peripheral cytoplasmic staining for: (**c**) Anti-CuZnSOD (*arrow*); (**e**) Anti-GPX; (**f**) Anti-GST-P; and (**g**) Anti-GST-L. Stromal cells of tumor showed light labeling with antibodies to glutathione dependent enzymes (*arrows*, **e–g**). For comparison to the cell of origin, see proximal tubule in Fig. 1. Tumor cells did not show significant staining with normal rabbit serum (NRS) (**h**). a–h, × 700

ants showed lipid droplets and glycogen in the cytoplasm, a few peripherally located small mitochondria, microvilli at the cell surface, and basement membrane outside the cells. Granular cell variants had numerous medium-sized mitochondria located throughout the cell cytoplasm, and the cell cytoplasm did not demonstrate significant lipid or glycogen. The cell surface of the granular variant also showed microvilli, and there was basement membrane outside the cells. Mixed tumors showed varying proportions of these two cell types.

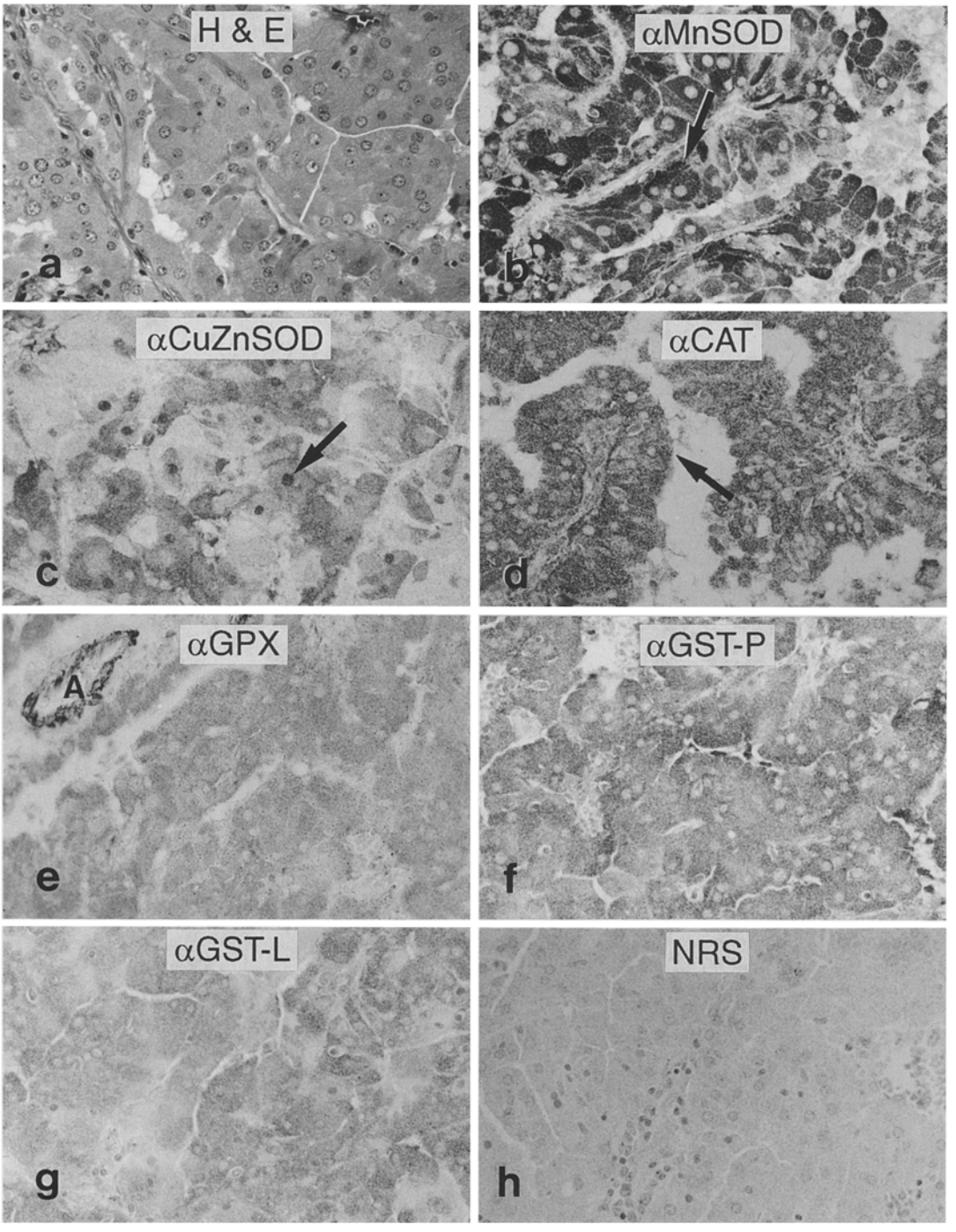

Fig. 3 Immunogold staining of a granular cell variant of human renal cell adenocarcinoma. (**a**) Hematoxylin and eosin (H and E) stain. Tumor cells were arranged in nests or glands. The nucleus was relatively small and the cytoplasm was granular. Tumor cells showed intense diffuse cytoplasmic staining (*arrows*) for: (**b**) Anti-MnSOD and (**d**) Anti-CAT. These cells showed light diffuse cytoplasmic labeling for: (**c**) Anti-CuZnSOD; (**e**) Anti-GPX; (**f**) Anti-GST-P; and (**g**) Anti-GST-L. Nuclei stained heavily with antibody to CuZnSOD (**c**, *arrow*). As an internal control with anti-GPX (**e**), a small renal artery (A) showed significant labeling, while the tumor exhibited light staining. For comparison to cell of origin, see proximal tubule in Fig. 1. (**h**). Tumor cells did not show significant staining with normal rabbit serum (NRS). **a–h**, × 700

Light microscopic analysis of antioxidant enzyme staining patterns in normal kidney and renal cell carcinomas

Enzyme staining patterns were studied in the cell of origin of the renal cell carcinoma, the proximal tubule. The proximal tubule showed heavy granular cytoplasmic staining for MnSOD (Fig. 1b), intense nuclear and diffuse cytoplasmic staining for CuZnSOD (Fig. 1c), heavy granular cytoplasmic labeling for CAT (Fig. 1d), heavy granular and diffuse cytoplasmic staining for

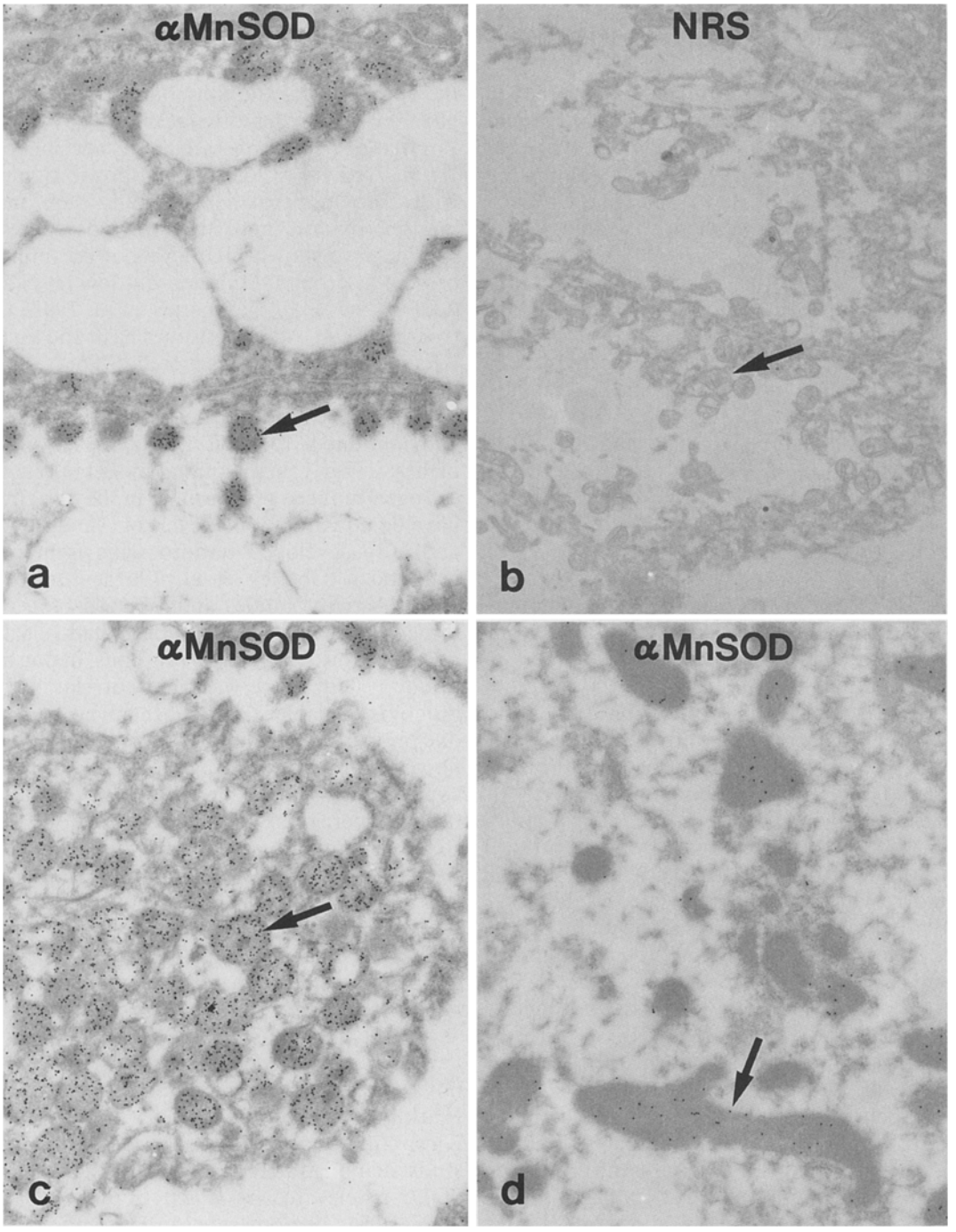

Fig. 4 Ultrastructural immunogold analysis of human kidney cells using antibody to MnSOD. (**a**) Clear cell variant of renal adenocarcinoma. Small oval mitochondria located along the periphery of the cell showed light staining for MnSOD (*arrow*). (**b**) Clear cell variant of renal adenocarcinoma. Normal rabbit serum (NRS) control. Mitochondria did not exhibit labeling (*arrow*). (**c**) Granular cell variant of renal adenocarcinoma. Numerous medium-sized oval mitochondria located throughout the cytoplasm showed moderate-heavy staining for MnSOD (*arrow*). (**d**) Normal human kidney proximal tubule. Numerous large branching mitochondria located at the base of the proximal tubular cells showed light staining for MnSOD (*arrow*). **a–d**, ×19 200

GPX (Fig. 1e), heavy diffuse cytoplasmic staining for GST-P isolate (Fig. 1f), and strong nuclear and diffuse cytoplasmic labeling for GST-L isolate (Fig. 1g).

Clear cell adenocarcinomas of the kidney demonstrated low staining for all AEs (compare proximal tubule in Fig. 1 to clear cell adenocarcinoma in Fig. 2). All clear cell variants ($n=14$) showed a similar staining pattern. The granular cell type of renal cell adenocarcinoma showed low staining of all enzymes studied except MnSOD and CAT (Fig. 3); these two enzymes labeled heavily in granular cells. All tumors composed of granular cell variants ($n=4$) or mixed clear and granular

cell tumors ($n=7$) demonstrated identical labeling characteristics of granular all types. In mixed cell tumors, all clear cells had low level of staining for AEs, while all granular cells had high levels of MnSOD and CAT. The distribution of staining for the granular cell type of renal adenocarcinoma was different than in the cell of origin; the proximal tubule showed heavy granular staining for both MnSOD and CAT, while the staining in the granular variant was diffuse for both of these antibodies (compare Fig. 3 to Fig. 1).

In all cases analyzed in this study, adjacent noninvolved renal parenchyma showed the same staining pattern as normal kidney; importantly, adjacent proximal tubules exhibited moderate to heavy labeling for all AEs at the same time that tumors showed little staining. These positive internal controls suggested that negative staining of tumors indicated that they had very little immunoreactive protein.

Immunogold electron microscopy

Ultrastructural analysis of clear cell adenocarcinomas demonstrated infrequent small peripheral mitochondria, while the granular cell type showed numerous medium-sized mitochondria arranged diffusely throughout the cytoplasm. The mitochondria of the granular cell type were larger than those of the clear cell type. Electron microscopy was performed in all of the renal adenocarcinomas used for histologic analysis in this study and they all showed the mitochondrial morphology described above. In contrast, proximal tubules from normal kidney exhibited extremely large branching mitochondria which were often located in the basal cytoplasm of the cell. The relationships summarized above for mitochondrial morphology, size, and number are illustrated in Fig. 4 in the ultrastructural immunogold analysis described below. Relative mitochondrial size, number, and morphology can be directly compared in Fig. 4 since these micrographs are all at the same magnification.

In order to better understand the altered staining patterns in renal adenocarcinomas compared to their cell of origin (the proximal tubule), we used ultrastructural immunogold techniques to study the distribution of this enzyme in normal and neoplastic tissue. Using immunogold techniques, all of these cell types stained with anti-MnSOD (Fig. 4), although the mitochondria of the granular variant of renal adenocarcinoma appeared to have the highest labeling density. All clear cell tumors examined had the same ultrastructural immunogold staining pattern ($n=3$); similarly, all 3 granular cell tumors studied had the same labeling pattern. The 1 mixed cell tumor examined demonstrated clear cells with infrequent small lightly labeled mitochondria, while granular cells in the same tumor showed numerous medium-sized heavily labeled mitochondria. No significant immunolabeling was observed in the sections prepared under control conditions.

Discussion

The reliability of the present results depends largely on the specificity of the antibodies used for analysis. We have previously demonstrated using Western blot analysis that our antibodies are highly specific (Oberley et al. 1993). Activity studies have shown tight correlation with immunoperoxidase results. For instance, immunoperoxidase and immunogold studies in several species have shown high levels of AE immunoreactive protein in proximal tubules, but low levels in glomeruli (Oberley et al. 1990; Oberley et al. 1993; Oberley and Oberley 1994). Activity studies have shown low levels of AEs in glomeruli (Steinert et al. 1986; Yang et al. 1988), but high activities of AEs in proximal tubules (Yang et al. 1987). Therefore, in normal kidney, activity studies and immunohistochemical studies are in close agreement. Similarly, in an animal model of renal cancer, the estrogen-induced renal cancer in the Syrian hamster, we have demonstrated low levels of AEs in both early foci and fully developed tumors using immunoperoxidase techniques (Oberley et al. 1991a; McCormick et al. 1991). Activity studies and Western blot analysis confirmed that hamster renal tumors had very low levels of AEs (McCormick et al. 1991). Thus, in one animal renal tumor system, there was tight correlation between immunohistochemical and biochemical analysis. Finally, one previous detailed study of a single human renal adenocarcinoma showed high MnSOD activity but low copper, zinc superoxide dismutase, catalase, and glutathione peroxidase activites (Yang et al. 1987), results in agreement with some of the tumors described in the present study using immunogold techniques. One further point of note is that staining of adjacent normal tissue showed the same pattern as that previously described in normal fresh human kidney tissue (Oberley and Oberley 1994), thereby providing an internal control for the immunogold tumor results.

Although AEs are generally low in animal tumors, a few recent studies have suggested that low AE activity is not a general marker of human neoplasia (Westman and Marklund 1981; Yang et al. 1987; Nakata et al. 1992; Kawamura et al. 1992; Iizuka et al. 1984). The present study demonstrated that the activity of one AE, MnSOD, is elevated in some human renal adenocarcinomas. As demonstrated in immunogold studies, one major reason for the extreme variability in MnSOD activities may be that renal adenocarcinomas contain varying proportions of clear and granular cell types. Clear cell types have low levels of MnSOD immunoreactive protein, while granular cells have high levels. The present study would have been enhanced by simultaneous analysis of AE activities and immunogold analysis in the same tumors. This was not possible since the study was performed on a retrospective basis with frozen or fixed tissues. Future studies will perform activity and immunogold analysis on fresh tissues.

One cell type, the granular cell type of renal cell carcinoma, demonstrated heavy cytoplasmic labeling for

MnSOD and CAT. This result demonstrated that AEs are not always lower in neoplastic tissue. However, this result does not imply that MnSOD levels were normal in this tumor. Indeed, the granular cell variant of renal adenocarcinoma showed greatly increased levels of immunoreactive protein. Recent studies have demonstrated that normal cells must have an optimal level of SOD; increased or decreased levels may both lead to cell damage (Avraham et al. 1988; Elroy-Stein et al. 1988). Immunogold ultrastructural analysis showed that MnSOD in the granular cell variant was present in large amounts in an increased number of medium-sized mitochondria (compared to normal proximal tubule), suggesting an abnormal regulation of MnSOD in this tumor. In general, when MnSOD is induced, the number of mitochondria remains the same, but the amount of MnSOD per mitochondria increases (Oberley et al. 1989). The present study documents abnormal mitochondrial size (decreased in both clear cell and granular cell variants of renal adenocarcinoma) and number (decreased in clear cell variant; increased in granular cell variant) in renal cancer compared to the cell of origin, the proximal tubule, and altered levels of MnSOD in these tumors (clear cell variant, low immunoreactive protein; granular cell variant, high immunoreactive protein). Activity data in Table 1 showed some renal cell carcinomas with either elevated or depressed activities compared to normal kidney, but some tumors showed activity similar to normal kidney homogenates. One can explain this data in at least one way by postulating that those with lowered activity were clear cell variants, those with elevated activity were granular cell variants, while those tumors with activities similar to normal kidney were mixed tumors composed of clear and granular cells. Prospective studies, rather that utilization of retrospective analysis as described in the present study, will allow us to determine whether this hypothesis is true.

The distinctive patterns of immunogold labeling observed in the present study suggest the possible utility of antibodies to AEs as possible diagnostic markers. Future studies will assess the levels of antioxidant enzymes in all types of renal tumors. Of especial interest will be whether immunogold analysis will allow distinction between hyperplasia/adenoma/malignancy. Several studies have shown that the granular cell variant of renal cell carcinoma has a worse prognosis than that of the clear cell variant (reviewed in Fromowitz and Bard 1990). One possible consequence of elevated antioxidant enzymes is resistance to chemotherapeutic agents and radiotherapy (Hirose et al. 1993). Future studies will try to resolve whether increased AEs correlates with a poorer prognosis, and could in fact explain the poorer survival with the granular cell variant of renal cell carcinoma.

ROS species are known to cause cell damage (Sies and Cadenas 1985), mutations (Moraes et al. 1991), and may cause cancer (Kennedy et al. 1984). On the other hand, ROS can cause cell proliferation (Armato et al. 1984; Shibanuma et al. 1988; Oberley 1985; Burdon and Rice-Evans 1989; Murrell et al. 1990; Craven et al. 1986), cell differentiation (Allen and Balin 1989), and can affect gene transcription (Schreck and Bauerle 1991; Nose et al. 1991). Therefore, regardless of how one interprets the role of AEs in cancer, the changes in AEs described in this report have dramatic consequences for the cell. All of the tumors examined had lowered staining for most of the AEs. Understanding the significance of these changes for the cancer cell may lead to a better understanding of the carcinogenic process.

In conclusion, this study shows extreme variablity in manganese superoxide dismutase activities in human renal cell carcinomas. Immunogold analysis suggested that one possible explanation for this variability is that granular cells of renal cell carcinomas contain high levels of manganese superoxide dismutase, and many tumors contain varying proportions of granular cells. Ultrastructural immunogold analysis confirmed a large amount of immunoreactive protein in granular cells; this protein was localized in mitochondria that were much smaller than those present in the cell of origin of this neoplasm, the renal proximal tubule. Future studies will be aimed at studying the utility of AEs as markers for subclassifying the several types of human renal neoplasms.

Acknowledgements This research was supported in part by a Merit Review grant from the Medical Research Service of the Department of Veterans Affairs and by grant number CA 41267 from the National Institutes of Health.

References

Allen RG, Balin AK (1989) Oxidative influence in development and differentiation: an overview of a free radical theory of development. Free Radic Biol Med 6:631–661

Armato U, Andreis PG, Romano F (1984) Exogenous Cu, Zn superoxide dismutase suppresses the stimulation of neonatal hepatocytes' growth by tumor promoters. Carcinogenesis 51:1547–1555

Avraham KB, Schickler M, Sapoznikov B, Yaron R, Groner Y (1988) Down's syndrome: abnormal neuromuscular junction in tongue in transgenic mice with elevated levels of human Cu/Zn-superoxide dismutase. Cell 54:823–829

Bravard A, Sabatier L, Hoffschir F, Ricoul M, Luccioni C, Dutrillaux B (1992a) SOD 2: a new type of tumor suppressor gene. Int J Cancer 51:476–480

Bravard A, Hoffschir F, Sabatier L, Ricoul M, Pinton A, Cassingena R, Estrade S, Luccioni C, Dutrillaux B (1992b) Early superoxide dismutase alterations during SV40 induced transformation of human fibroblasts. Int J Cancer 52:797–801

Burdon RH, Rice-Evans C (1989) Free radicals and the regulation of mammalian cell proliferation. Free Radic Res Commun 6:345–358

Church SL, Grant JW, Ridnour LA, Oberley LW, Swanson P, Meltzer PS, Trent JM (1993) Increased manganese superoxide dismutase expression suppresses the malignant phenotype of cultured human melanoma cells. Proc Natl Acad Sci USA 90:3113–3117

Coles B, Ketterer B (1990) The role of glutathione and glutathione transferases in chemical carcinogenesis. Crit Rev Biochem Mol Biol 25:47–70

Coursin DB, Cihla HP, Oberley TD, Oberley LW (1992) Immunolocalization of antioxidant enzymes and isozymes of glu-

tathione S-transferase in normal rat lung. Am J Physiol 263:2679–2691
Craven PA, Pfanstiel J, DeRubertis FR (1986) Role of reactive oxygen in bile salt stimulation of colonic epithelial proliferation. J Clin Invest 77:850–857
Elroy-Stein O, Bernstein Y, Groner Y (1988) Overproduction of human Cu/Zn-superoxide dismutase in transfected cells: extenuation of paraquat-mediated cytotoxicity and enhanced lipid peroxidation. EMBO J 5:615–622
Fromewitz FB, Bard RH (1990) Clinical implications of pathologic subtypes in renal all carcinoma. Sem Urol VIII:31–50
Gonzalez A, Oberley TD, Li JJ (1989) Morpholgical and immunohistochemical studies of the estrogen-induced Syrian hamster renal tumor: probable cell of origin. Cancer Res 49:1020–1028
Hirose K, Longo DL, Oppenheim JJ, Matsushima K (1993) Overexpression of mitochondrial manganese superoxide dismutase promotes the survival of tumor cells exposed to interleukin-1, tumor necrosis factor, selected anticancer drugs, and ionizing radiation. FASEB J 7:361–368
Iizuka S, Taniguchi N, Makita A (1984) Enzyme-linked immunosorbent assay for human manganese-containing superoxide dismutase and its content in lung cancer. J Natl Cancer Inst 72:1043–1049
Kawamura N, Suzuki K, Ishikawa M, Iizuka S, Miyake M, Mino M, Taniguchi N (1992) High levels of Mn-superoxide dismutase in serum of patients with neuroblastoma and in human neuroblastoma cell lines. Free Radic Biol Med 12:281–286
Kennedy AR, Troll W, Little JB (1984) Role of free radicals in the initiation and promotion of radiation transformation in vitro. Carcinogenesis 5:1213–1215
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
McCormick ML, Oberley TD, Elwell JH, Oberley LW, Sun Y, Li JJ (1991) Superoxide dismutase and catalase levels during estrogen-induced renal tumorigenesis, in renal tumors and their autonomous variants in the Syrian hamster. Carcinogenesis 12:977–983
Moraes EC, Keyse SM, Tyrell RM (1991) Mutagenesis by hydrogen peroxide treatment of mammalian cells: a molecular analysis. Carcinogenesis 11:283–293
Murrell GAC, Francis MJD, Bromley L (1990) Modulation of fibroblast proliferation by oxygen free radicals. Biochem J 265:659–665
Nakata T, Suzuki K, Fujii I, Ishika M, Tatsumi H, Sugiyami T, Nishida T, Shimuzu T, Yakushiji M, Taniguchi N (1992) High expression of manganese superoxide dismutase in 7,12-dimethylbenz[a]anthracene-induced ovarian cancer and increased serum levels in the tumor-bearing rats. Carcinogenesis 13:1941–1947
Nose K, Shibanuma M, Kikachi K, Hageyama H, Sakiyama S, Kuroki T (1991) Transcriptional activation of early-response genes by hydrogen peroxide in a mouse osteoblastic cell line. Eur J Biochem 201:99–106
Oberley TD (1985) The possible role of reactive oxygen metabolites in cell division. In: Oberley LW (ed) Superoxide dismutase, Vol. III. CRC, Boca Raton, Fla pp 83–98
Oberley LW, Oberley TD (1986) Free radicals, cancer, and aging. In: Johnson JE (ed) Free radicals, aging, and degenerative diseases. Alan R. Liss, Inc., New York, pp 325–371
Oberley LW, Kasemset-St. Clair D, Autor AP, Oberley TD (1987) Increase in manganese superoxide dismutase activity in the mouse heart after x-irradiation. Arch Biochem Biophys 254:69–80
Oberley LW, Ridnour LA, Sierra-Rivera E, Oberley TD, Guernsey DL (1989) Superoxide dismutase activities of differentiating clones from an immortal cell line. J Cell Physiol 138:50–60
Oberley TD, Oberley LW, Slattery AF, Lauchner LJ, Elwell JH (1990) Immunohistochemical localization of antioxidant enzymes in adult Syrian hamster tissues and during kidney development. Am J Pathol 137:199–214
Oberley TD, Gonzalez A, Lauchner LJ, Oberley LW, Li JJ (1991a) Characterization of early kidney lesions in estrogen-induced tumors in the Syrian hamster. Cancer Res 51:1922–1929
Oberley TD, Oberley LW, Slattery AF, Elwell JH (1991b) Immunohistochemical localization of glutathione-S-transferase and glutathione peroxidase in adult hamster tissues and during kidney development. Am J Pathol 139:355–369
Oberley TD, Coursin DB, Cihla HP, Oberley LW, El-Sayyad N, Ho YS (1993) Immunolocalization of manganese superoxide dismutase in normal and transgenic mice expressing the human enzyme. Histochem J 25:267–279
Oberley TD, Oberley LW (1994) Localization of antioxidant enzymes during hamster kidney development. In: Davies, KA (ed) Oxygen '93. Pergamon Press, Oxford New York (in press)
Schreck R, Bauerle PA (1991) A role for oxygen radicals as second messengers. Trends Cell Biol 1:39–42
Shibanuma M, Kuroki T, Nose K (1988) Induction of DNA replication and expression of proto-oncogenes c-myc and c-fos in quiescent Balb/3T3 cells by xanthine-xanthine oxidase. Oncogene 3:17–21
Sies H, Cadenas E (1985) Oxidative stress: damage to intact cells and organs. Philos Trans R Soc Lond Biol 311:617–631
Spitz DR, Oberley LW (1989) An assay for superoxide dismutase activity in mammalian tissue homogenates. Anal Biochem 179:8–18
Spitz DR, Elwell JH, Sun Y, Oberley LW, Oberley TD, Sullivan SJ, Roberts RJ (1990) Oxygen toxicity in control and H_2O_2-resistant Chinese hamster fibroblast cell lines. Arch Biochem Biophys 277:249–260
St. Clair DK, Wan XS, Oberley TD, Muse KE, St. Clair WH (1992) Suppression of radiation-induced neoplastic transformation by overexpression of mitochondrial superoxide dismutase. Mol Carcinog 6:238–242
Steinert BW, Anderson PJ, Oberley LW, Oberley TD (1986) Kidney glomerular explants in serum-free media: demonstration of intracellular antioxidant enzymes and active oxygen metabolites. In Vitro Cell Dev Biol 22:285-294
Towbin H, Staehelin R, Gordon J (1976) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354
Westman NG, Marklund SL (1981) Copper and zinc-containing superoxide dismutase and manganese containing superoxide dismutase in human tissues and human malignant tumors. Cancer Res 41:2962–2966
Yang AH, Oberley TD, Oberley LW, Schmid SM, Cummings KB (1987) In vitro modulation of antioxidant enzymes in normal and malignant renal epithelium. In Vitro Cell Dev Biol 23:546–558
Yang AH, Oberley TD, Oberley LW, Ramanathan R (1988) Effect of cell substrate on antioxidant enzyme activities in cultured renal glomerular epithelium. Am J Pathol 130:616–628