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Original Contribution

LOCALIZATION OF SUPEROXIDE DISMUTASE ACTIVITY IN RAT TISSUES

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Abstract—Superoxide anion radicals have been implicated in a variety of pathological processes. Under physiological conditions, superoxide dismutase (SOD) is effectively able to disproportionate superoxide anions into hydrogen peroxide and dioxygen. Until now, no techniques have been available to localize SOD activity within tissues. In the present study, SOD activity was detected in different rat tissues using a thin film of xanthine oxidase between the glass slide and the unfixed cryostat section and a medium containing hypoxanthine as a source of electrons for the production of superoxide anions. The incubation medium also contained cerium ions to precipitate the hydrogen peroxide product and polyvinyl alcohol to prevent leakage of soluble and/or loosely bound enzymes from the sections into the incubation medium. The cerium perhydroxides that are formed were visualized for the light microscope in a second step using an incubation medium consisting of 3,3′-diaminobenzidine, cobalt ions, and hydrogen peroxide, which results in oxidation of the diaminobenzidine to the final insoluble blue reaction product. By this methodology, high enzyme activity was found not only in endothelial cells of liver and kidney but also in hepatocytes of liver, myocytes of heart, smooth and striated cells of muscle, acinar cells of pancreas, epithelial cells of kidney ducts, and epithelial cells of the small intestine and colon. These findings were largely in agreement with immunohistochemical data obtained using antibodies against the Cu/Zn- and Mn-SODs. However, high activity was also detected extracellularly at the surface of epithelia of trachea, esophagus, small intestine, and colon and at the extracellular matrices, cartilage, and connective tissues. We conclude from these latter data that the activity of the extracellular form of the dismutase is localized. The present method allows the analysis of all three types of known SOD activity (Cu/Zn, Mn, and extracellular) in different tissues and cell compartments. *Copyright © 1996 Elsevier Science Inc.*

Keywords—Superoxide dismutase activity, Cerium, Enzyme histochemistry, Extracellular superoxide dismutase, Superoxide anion, Free radical

INTRODUCTION

The superoxide anion radical is generated by the one-electron reduction of dioxygen. In cells and tissues, these radicals are produced by enzymes such as xanthine oxidase in the cytoplasm, the electron transport chain in mitochondria, and NAD(P)H oxidase at the plasma membrane of phagocytes.[1,2]

Superoxide anion radicals have been implicated in a variety of pathological processes.[3–6] Under physiological conditions, superoxide dismutase (SOD; EC 1.15.1.1) is effectively able to dismutate or disproportionate superoxide anions into hydrogen peroxide and dioxygen.[7] Biochemical studies have shown that SOD activity is present in all tissues, with the highest activity in liver and kidney. Activity has also been shown to vary with species in certain tissues.[8–10]

At least three different forms of SOD have been described in mammalian tissues: cytosolic copper-zinc SOD (Cu/Zn-SOD), mitochondrial manganese SOD (Mn-SOD),[11] and the high molecular weight extracellular SOD (EC-SOD).[12] Immunohistochemical analyses of different rat and canine tissues revealed the presence of abundant Cu/Zn-SOD protein in hepatocytes, epithelial cells of proximal tubules in kidney, striated muscle cells, oligodendrocytes in glia of the central nervous system, islets of Langerhans, and epi-

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thelial cells of large bowel and bronchi.[13] Dobashi et al.[14] investigated the immunohistochemical localization of both Cu/Zn- and Mn-SOD in various rat tissues. These authors found localization patterns for Cu/Zn-SOD similar to those found by Thaete et al.[13] In addition, Cu/Zn-SOD was observed in epithelial cells of pancreatic ducts, distal tubules, and collecting ducts in kidney, as well as in myocardial cells.[14] The Mn-SOD was localized in cells that also expressed Cu/Zn-SOD.[14] As discussed by Van Noorden and Frederiks,[15] immunohistochemical localization of enzymes does not per se give information on the activity of enzymes, and biochemical analysis of enzyme activities does not yield information on the cellular localization of the activity. Therefore, it would be useful to develop a technique to demonstrate SOD activity in tissues in situ.

In the present study, we have localized SOD activity after generating oxygen radicals as substrates for SOD in a reaction medium containing hypoxanthine and xanthine oxidase. One product of the dismutase-catalyzed reaction, H_2O_2, reacts with cerium ions, giving rise to an insoluble reaction product.[16,17] This system was originally developed for electron microscopical analysis of SOD activity in alveolar macrophages.[18,19] However, we have expanded this system and developed methodology to visualize and hence to localize SOD activity using the light microscope. This is accomplished when the cerium perhydroxides formed oxidize the diaminobenzidine in a second incubation step to form the final insoluble blue reaction product.[20-22]

MATERIALS AND METHODS

Three-month-old male Wistar rats (200–250 g; TNO, Zeist, The Netherlands) were exposed to a controlled dark–light cycle (light 7:00 A.M. to 7:00 P.M.) throughout the acclimatization period of at least 1 week. The animals were sacrificed under ether anesthesia between 9:00 and 10:00 A.M. to avoid possible chronobiological variations. Liver, kidney, heart, pancreas, trachea, esophagus, small intestine, and large intestine were removed as quickly as possible. Tissues were cut into pieces up to 0.5 cm^3 and frozen in liquid nitrogen in closed vials.[15] Tissue blocks were stored at −80°C until further use. Cryostat sections (8 μm thick) were cut at −25°C on a motor-driven Bright cryostat fitted with a retraction microtome at a constant low speed to obtain sections of constant thickness.

Xanthine oxidase was prepared on clean glass slides by spreading 10 μl of an aqueous solution containing 1–50 mIU xanthine oxidase (phosphate-free, isolated from cow milk; Boehringer, Mannheim, Germany) onto the slide over an area of approximately 1 cm^2. These enzyme films on the slides were air-dried for 15 min at room temperature (22°C). Tissue sections from the cryostat knife were mounted on top of the enzyme film[15,23] and air-dried for 5 min at room temperature before the incubation medium to demonstrate SOD activity was added for 15 min at 37°C. The composition of the incubation medium was based on that described for the histochemical assay of xanthine oxidase[24] and contained 10% (w/v) polyvinyl alcohol (PVA; weight average M_r 70,000–100,000; Sigma, St. Louis, MO, USA), 100 mM Tris-maleate buffer, pH 7.6, 10 mM cerium chloride (Fluka Chemie A.G., Buchs, Switzerland), and 0.1–1.0 mM hypoxanthine (Merck, Darmstadt, Germany). After incubation in the PVA–hypoxanthine medium, sections were rinsed in hot distilled water (60°C) to stop the reaction immediately and to remove the viscous incubation medium. The final step for visualization was performed by incubating tissue sections for 30 min at room temperature in 50 mM sodium acetate buffer at pH 5.3 with 42 mM cobalt chloride, 100 mM sodium azide, 1.4 mM 3,3′-diaminobenzidine (Sigma), and 0.6 mM H_2O_2[20]. After rinsing, the sections were embedded in glycerol jelly and immediately analyzed and/or photographed using the light microscope and stored in the dark at 4°C to minimize fading of the final reaction product.[20,26]

The optimized incubation conditions of 15 mIU xanthine oxidase in the film and 0.5 mM hypoxanthine in the medium were applied on the unfixed cryostat sections of rat liver to study the specificity of the histochemical reaction to demonstrate SOD activity. Control incubations were performed in the absence of hypoxanthine, in the absence of exogenous xanthine oxidase, or in the absence of hypoxanthine and xanthine oxidase. Another control was the incubation that lacked hypoxanthine and in which exogenous xanthine oxidase was replaced by bovine serum albumin. Incubations were also performed with optimal media saturated with pure oxygen or pure nitrogen using a tonometer to avoid gas bubbles in the viscous PVA-containing medium.[15] The inhibitor diethyldithiocarbamate (10 mM) was added to the incubation medium in the presence of hypoxanthine and xanthine oxidase.[12] The possible involvement of catalase activity in the histochemical reaction was tested by adding 10 mM 3-amino-1,2,4-triazole to the incubation medium to inhibit catalase activity.[25] The enzymatic character of the reaction was studied by incubating sections with increasing incubation times (0–60 min) in the PVA-hypoxanthine media.

The immunohistochemical detection of Cu/Zn- and Mn-SOD was performed to compare the localization of SOD activity by this new method with the presence of the proteins using serial sections of the same material. Cryostat sections were air-dried for at least 60 min at

room temperature, after which they were fixed in 4% (w/v) formaldehyde in 0.01 M phosphate-buffered saline (PBS) at pH 7.4. Immunohistochemical reactions were performed by incubation with sheep anti-human Cu/Zn-SOD or Mn-SOD polyclonal antibodies (The Binding Site, Birmingham, England), followed by incubation with horseradish peroxidase labeled rabbit anti-sheep immunoglobulins. Afterward, these sections were stained for peroxidase activity with 3,3′-diaminobenzidine. Finally, after rinsing in distilled water, sections were counterstained with hematoxylin for 1 min and rinsed thoroughly before mounting in glycerol jelly (Van Driel, personal communication).

RESULTS

The highest amounts and a homogeneous distribution of final insoluble reaction product over entire cryostat sections of rat liver were obtained when a film containing xanthine oxidase at 15 mIU/10 μl was applied per section and when 0.5 mM hypoxanthine was present in the incubation medium. Increasing periods of incubation in the presence of hypoxanthine and xanthine oxidase resulted in increasing amounts of final reaction product, but the optimal time was found to be 15 min. The insoluble reaction product was found in rat liver in sinusoidal cells, liver parenchymal cells, endothelial cells of large blood vessels, and cellular and extracellular elements of the portal tract (Fig. 1; Table 1). Outside the section, but inside the area of the film of xanthine oxidase, a small amount of bluish precipitate was produced, apparently due to the formation of H_2O_2 directly by xanthine oxidase.[27] In the absence of hypoxanthine and xanthine oxidase or in the absence of hypoxanthine and in the presence of exogenous xanthine oxidase, very little final reaction product was found. Similar findings were obtained when exogenous xanthine oxidase was replaced by bovine serum albumin. In the presence of hypoxanthine but in the absence of exogenous xanthine oxidase, small amounts of final reaction product were observed in sinusoidal endothelial cells and liver parenchymal cells. This was due to endogenous xanthine oxidase activity.[24] Incubations performed in media saturated with pure oxygen did not yield higher amounts of final reaction product, whereas incubations performed in the presence of nitrogen resulted in no formation of any final reaction product. Aminotriazole did not affect the amount of final reaction product formed, but diethyldithiocarbamate completely abolished its formation. The weak staining intensity in the presence of diethyldithiocarbamate was similar to that obtained after incubation in the absence of hypoxanthine and xanthine oxidase. Therefore, all these data taken together suggest that this new procedure is specific for demonstrating SOD activity in tissue sections.

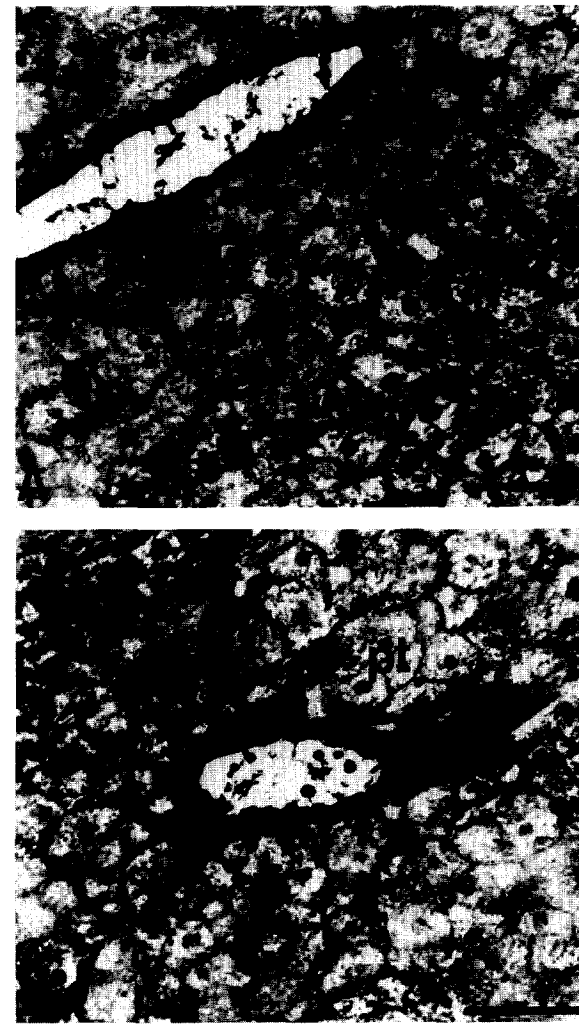

Fig. 1. Micrographs of an unfixed cryostat section of rat liver incubated for the demonstration of SOD activity. (A) Central vein (cv). (B) Portal tract (pt). Activity is present in liver parenchymal cells, sinusoidal cells, endothelial cells of large blood vessels as well as elements of the portal tract. Specific reaction product is also present in nuclei of liver parenchymal cells. Magnification for both is 300×; scale bar = 50 μm.

SOD activity was localized in other rat tissues as follows. In kidney, high SOD activity was found in epithelial cells of ducts of the medulla (collecting ducts and lis of Henle), while the brush border of epithelial cells of proximal tubules and epithelial cells of distal tubules in the cortex also showed activity (Table 1). All cellular and extracellular elements of glomeruli (endothelial cells, podocytes, mesangium cells, and extracellular matrix) showed high activity (Fig. 2). In heart tissue, high activity was detected on the fibrils in myocytes (Fig. 3), whereas endothelial cells did not show activity. In the pancreas, activity was localized at the

Table 1. Localization of Superoxide Dismutase Activity vs. Immunohistochemical Detection of Cu/Zn-SOD and Mn-SOD in Different Cell Types and Tissues of 3-Month-Old Male Wistar Rats

	SOD Activity	Cu/Zn-SOD	Mn-SOD
Liver			
Parenchymal cells	++	++	+
Sinusoidal cells	++	−	−
Endothelial cells	+++	−	−
Connective tissue	+++	−	−
Kidney			
Proximal tubules	++	+	+
Distal tubules	++	+	+
Collecting tubules	++	+	+
Lis of Henle	−+	+	+
Glomeruli	+++	−	−
Heart			
Myocytes	++	++	+
Endothelial cells	−	−	−
Pancreas			
Acinar cells	++	++	++
Collecting ducts	+	++	++
Islets of Langerhans	−	++	++
Trachea			
Epithelial cells			
Ciliary cells	−	++	++
Goblet cells	−	++	++
Basal cells	−	++	++
Connective tissue			
Fibroblasts	+	−	−
Macrophages	+	−	−
Extracellular matrix	+	−	−
Cartilage			
Chondrocytes	−	+	+
Extracellular matrix	++	−	−
Smooth muscle cells	+	±	±
Esophagus			
Epithelial cells			
Superficial	++	−	−
Intermediate	±	++	+
Basal	±	++	+
Mucous glands	+	+	+
Musculares externae			
Striated muscle cells	+	+	+
Small intestine			
Epithelial cells			
Enterocytes	+	++	+
Goblet cells	+	++	+
Colon			
Epithelial cells			
Resorptive cells	++	++	+
Goblet cells	+	++	+

−, Absent; ±, weak; +, moderate; ++, high; +++, very high.

basal side of acinar cells of the exocrine part of the gland, whereas the islets of Langerhans did not show any enzyme activity (Fig. 4). Epithelial cells of the pancreatic ducts also expressed activity. SOD activity was high at the surface of epithelial cells of the trachea, but could not be detected in epithelial cells (basal cells, ciliary cells, and goblet cells; Fig. 5). Activity was also present in other parts of the trachea, such as connective tissue, cartilage, and smooth muscle cells (Fig. 5). In the esophagus, little activity was present in living epithelial cells, but activity was largely present in the superficial layer composed of dead cells or cell remnants (Fig. 6). Activity was also present in other tissues of the esophagus, connective tissue of lamina propria and submucosa, mucous glands, smooth muscle cells, and striated muscle cells (Fig. 6). At the surface of epithelial cells and in epithelial cells of small intestine and colon, SOD was present, whereas in mucus it was absent (Figs. 7, 8). Cellular and extracellular elements of connective tissue and smooth muscle cells showed SOD activity (Figs. 7, 8). For comparison, the immunohistochemical localization of Cu/Zn- and Mn-SOD is presented in Table 1. Our findings are largely in

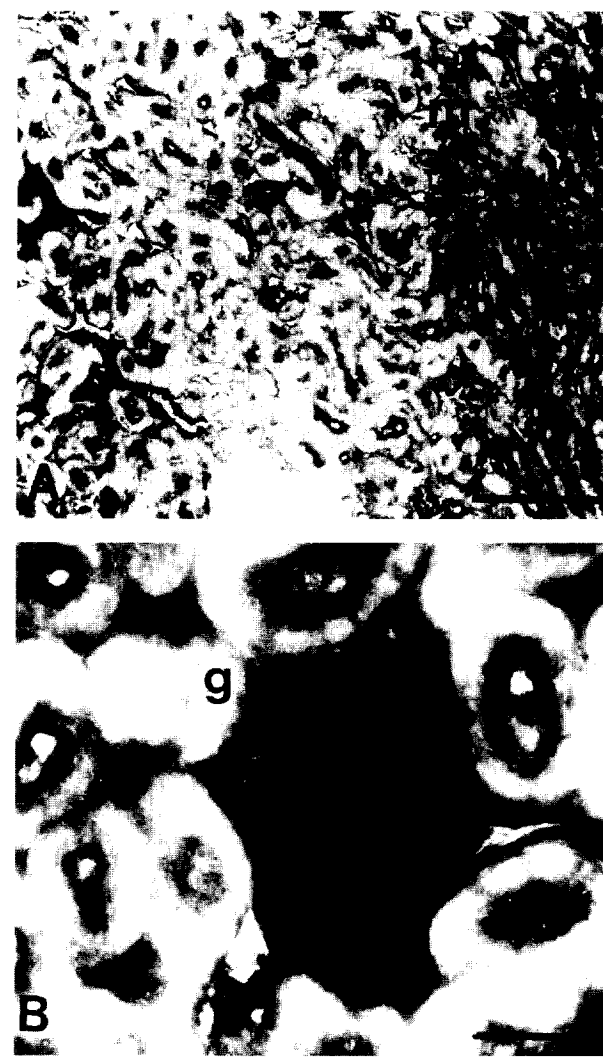

Fig. 2. Micrographs of an unfixed cryostat section of rat kidney incubated for the demonstration of SOD activity. (A) Medulla (m) at 75×; scale bar = 200 μm. (B) Glomerulus at 300×; scale bar = 50 μm. Activity is present in epithelial cells of ducts in the medulla, at the brush border of epithelial cells of proximal tubules in the cortex, and in glomeruli.

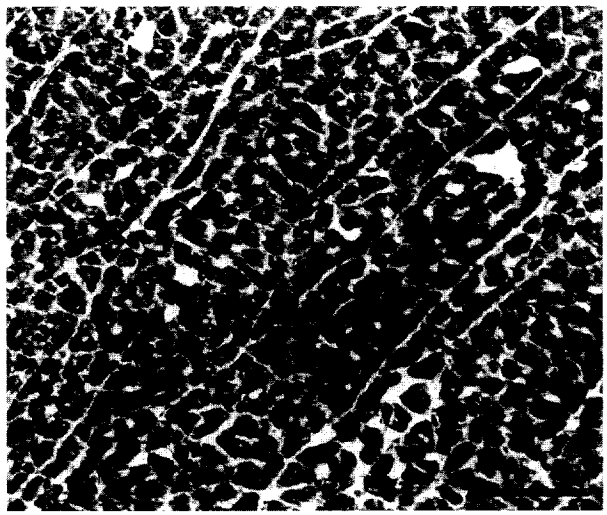

Fig. 3. Micrograph of an unfixed cryostat section of rat heart incubated for the demonstration of SOD activity. Activity is present at the myofibrils of myocytes. Magnification is 150×; scale bar = 100 μm.

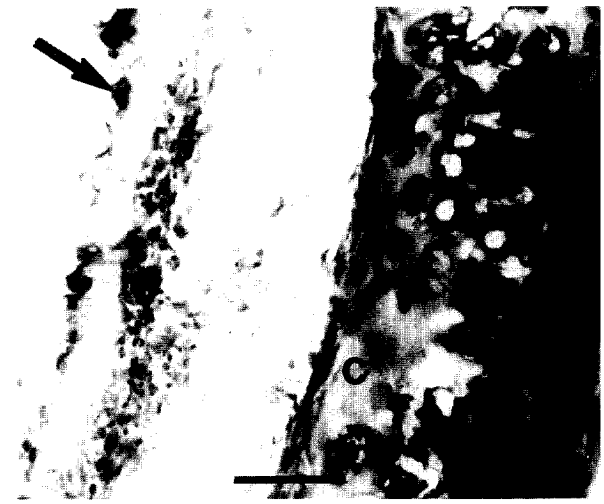

Fig. 5. Micrograph of an unfixed cryostat section of rat trachea incubated for the demonstration of SOD activity. Activity is present at the surface of epithelial cells (arrow) but not inside epithelial cells in connective tissue, cartilage (c), and smooth muscle cells. Magnification is 300×; scale bar = 50 μm.

agreement with those of Dobashi et al.[14] Moreover, similar localization patterns of SOD activity and the proteins were found for epithelial cells in different tissues. However, in some cases, the proteins were present without activity, as in islets of Langerhans of pancreas and in epithelial cells of trachea. In even more situations, SOD activity was present without detectable amounts of the Cu/Zn- and Mn-SOD proteins, as at the surfaces of epithelia and in the extracellular matrices, cartilage, and connective tissue.

DISCUSSION

The present study shows that SOD activity can be determined in unfixed cryostat tissue sections by using xanthine oxidase in a thin film on glass slides with hypoxanthine in an incubation medium, a system that produces superoxide anions as a substrate for SOD activity. As these anions are disproportionated to hydrogen peroxide and dioxygen, cerium ions precipitate the peroxide, which is visualized in an oxidation–reduction

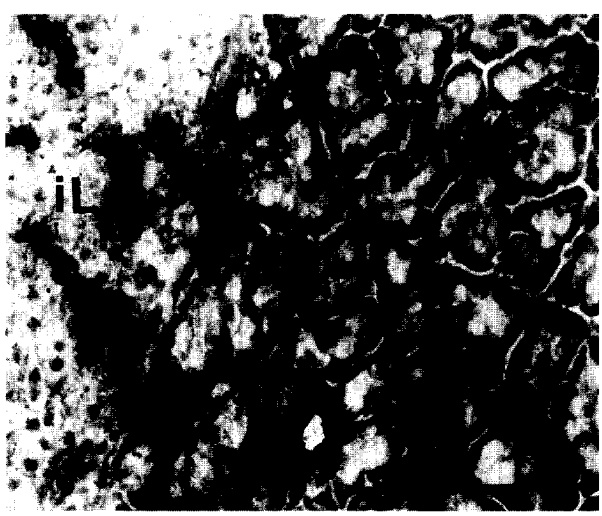

Fig. 4. Micrograph of an unfixed cryostat section of rat pancreas incubated for the demonstration of SOD activity. Activity is present at the basal side of acinar cells of the exocrine part and epithelial cells of the ducts, but not in the islets of Langerhans (iL). Magnification is 300×; scale bar = 50 μm.

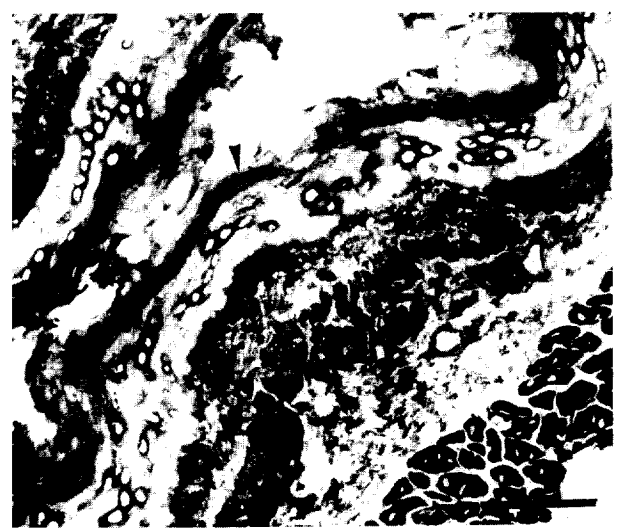

Fig. 6. Micrograph of an unfixed cryostat section of rat esophagus incubated for the demonstration of SOD activity. Activity is present in the superficial layer of the epithelium (arrowhead) but not in living epithelial cells in connective tissue, mucous glands, and striated muscle cells. Magnification is 150×; scale bar = 100 μm.

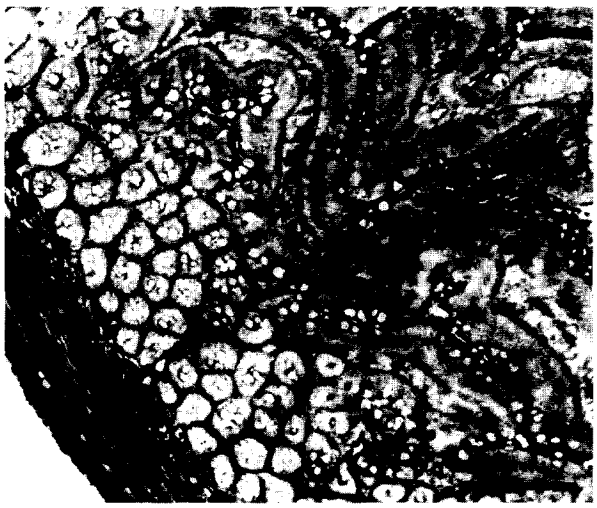

Fig. 7. Micrograph of an unfixed cryostat section of rat duodenum incubated for the demonstration of SOD activity. Activity is present at the surface of epithelial cells and inside epithelial cells but not in mucus in connective tissue and smooth muscle cells. Magnification is 75×; scale bar = 200 μm.

reaction producing a blue insoluble product from 3,3′-diaminobenzidine. Although some hydrogen peroxide is produced as well by xanthine oxidase, this causes only a low background staining inside and outside the tissue section that can be discriminated easily from the strong SOD-specific reaction. Under the optimized conditions of 15 mIU xanthine oxidase per section and 0.5 mM hypoxanthine in the incubation medium, superoxide anions are apparently produced for at least 1 h. Evidence for this comes from the fact that increasing amounts of final reaction product were found in rat liver tissue sections with increasing incubation times to 60 min. An optimal time of 15 min, however, is recommended for this new procedure. The absence of any detectable formation of final reaction product in the absence of hypoxanthine and xanthine oxidase, and in the absence of hypoxanthine but in the presence of exogenous xanthine oxidase, means that the concentration of endogenous hypoxanthine is not high enough to produce significant amounts of superoxide anions. The small amount of final reaction product formed in the presence of hypoxanthine but in the absence of exogenous xanthine oxidase is due to endogenous xanthine oxidase. However, this activity is very low in all tissues studied in comparison with SOD activity, an observation that is in accordance with the literature.[28] The specificity of this new procedure was established by the use of diethyldithiocarbamate, a specific inhibitor of all forms of SOD activity.[12] That the new procedure is specific for SOD is supported by the fact that saturation of media with nitrogen did not result in any formation of final reaction product. This is most likely due to the absence of dioxygen for superoxide anion production. The absence of any effect of aminotriazole on the formation of final reaction product means that catalase activity does not interfere in the trapping of hydrogen peroxide by cerium ions, a process that is essential in demonstrating the formation of hydrogen peroxide from the superoxide anions produced. This lack of effect of aminotriazole is easily understood when taking into account that catalase activity is exclusively present in peroxisomes,[29] whereas SOD activity is present in the cytoplasm and in mitochondria.[14]

The localization of SOD activity in the tissues investigated by the new method presented here is largely consistent with our immunohistochemical findings as well as those of Thaete et al.[13] and Dobashi et al.[14] For

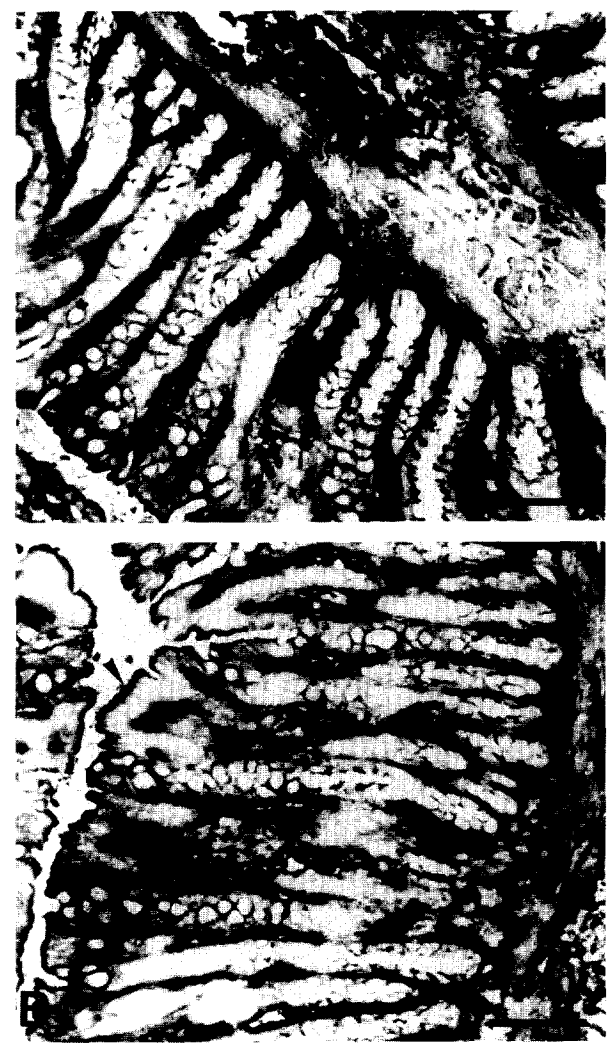

Fig. 8. Micrographs of an unfixed cryostat section of rat colon incubated for the demonstration of SOD activity. Activity is present at the surface of epithelial cells (arrowhead) and inside epithelial cells but not in mucus in connective tissue, and smooth muscle cells. Magnifications are 75× (A) and 150× (B); scale bars = 200 μm (A) and 100 μm (B).

example, it was shown that endothelial cells in liver and kidney contain high SOD activity (Figs. 1, 2), as do the epithelial cells of ducts in kidney and pancreas (Figs. 2, 4) and in small intestine and colon (Figs. 7, 8). On the other hand, epithelial cells of trachea and esophagus did not express activity (Figs. 5, 6). Moreover, SOD activity was present in hepatocytes, myocytes, and smooth and striated muscle cells (Figs. 1, 3, 5–8).

However, this new method indicates that some distinct differences from immunohistochemical data do exist. Immunohistochemically, the Cu/Zn-SOD and Mn-SOD proteins were found in epithelial cells of trachea and in islets of Langerhans (Table 1), whereas this new SOD activity procedure indicated that the SOD in these tissues was most likely not active (Fig. 4). It may be that the enzyme is not active inside these cells but is activated after secretion.

Another striking discrepancy was the high SOD activity found extracellularly on the surface of a number of epithelial cells (trachea, esophagus, duodenum, colon), in connective tissue (liver, trachea, esophagus, small intestine, colon), and in cartilage (trachea) as well as in glomeruli (kidney). Neither the protein Cu/Zn-SOD nor Mn-SOD could be detected immunohistochemically at these extracellular sites.[13,14] These data indicate that another dismutase, the extracellular form, has most likely been localized by this method. This enzyme is a tetrameric hydrophobic glycoprotein with a molecular weight of ~135,000.[30] It occurs not only in extracellular fluids but also inside tissues and cells. Marklund[31] investigated cultures of a large panel of human cell lines and found that fibroblasts and glia cells, but not endothelial cells, expressed activity of the extracellular form, which was secreted into the culture medium. This secreted enzyme showed a high affinity for heparin, and it was suggested that it reversibly binds to heparan sulfate proteoglycan ligands in the glycocalyx at the surface of most tissue cell types and in the interstitial matrix.[31] Only recently, Oury et al.[32] demonstrated this EC-SOD immunohistochemically in human lung. They showed the presence of EC-SOD in areas containing high amounts of type I collagen and other unidentified matrix elements and around the surface of vascular and airway smooth muscle cells. Sandström et al.[33] demonstrated that the EC-SOD is the less abundant dismutase of tissues. Our data show that a very high SOD activity was present extracellularly in comparison to intracellular SOD activity. This suggests that cells in contact with an external milieu (air or food), as well as cartilage and connective tissue, are well equipped with the antioxidant enzyme EC-SOD.

Whether the present histochemical procedure detects the cytoplasmic and/or mitochondrial SOD activity within cells cannot be conclusively assessed. The addition of polyvinyl alcohol to the incubation medium enables the demonstration of soluble and/or loosely bound enzymes as well as tightly bound enzymes.[34] Kuroda[18] demonstrated SOD activity in cytoplasm and mitochondria of alveolar macrophages at the electron microscopical level using fixed cells and an aqueous incubation medium, indicating that the activity of both forms can be detected. Electron microscopical studies are currently in progress to determine the precise intracellular localization of SOD activity with the present method.

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ABBREVIATIONS

SOD—superoxide dismutase
EC-SOD—extracellular superoxide dismutase