RESEARCH COMMUNICATION

Isolation and characterization of a rat cDNA clone encoding a secreted superoxide dismutase reveals the epididymis to be a major site of its expression

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Superoxide dismutase (SOD) plays a key role in combating loss of fertility of spermatozoa due to lipid peroxidation. Here we report the sequence of a cDNA encoding a secreted form of SOD isolated from a rat epididymal library. Northern-blot analysis indicates that the corresponding transcript is expressed principally in the cauda region of the epididymis, consistent with the

high levels of SOD enzyme activity found in cauda-epididymal plasma. Much lower levels of an identically sized transcript exist in all tissues examined, including placenta. PCR and subsequent sequence analysis of rat placental SOD strongly suggest that it is identical in sequence with epididymal SOD.

INTRODUCTION

Ubiquitous reactive oxygen species such as the superoxide anion, O_2^- , can elicit unprogrammed cell damage; indeed, O_2^- is believed to be a significant factor in the process of aging [1,2]. Mammalian spermatozoa are especially susceptible to oxygen free radicals and cytotoxic lipid hydroperoxides [3,4], since they contain an unusually high proportion of polyunsaturated phospholipids in their membranes. This toxicity results in increased membrane fragility, anatomical abnormalities and impaired fertility [5–7].

The levels of toxic oxygen free radicals and their deleterious lipid biproducts are normally regulated by the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPX). Although GPX has a ubiquitous tissue distribution, a unique secretory form [8] is expressed in the epididymis, a ductular organ that is contiguous with the testis and within which spermatozoa are modified and stored prior to ejaculation. Epididymal GPX transcripts are androgen-sensitive, can be detected in all regions of the epididymis and apparently encode a secreted enzyme whose function is to detoxify deleterious lipid hydroperoxides by reducing them to their corresponding alcohols at the expense of GSH. Like GPX, SOD exhibits a wide tissue distribution. This includes two intracellular forms of the enzyme, a Mn^{2+} -containing mitochondrial form (SOD2) and a cytosolic SOD (SOD1) containing an active-site Cu/Zn pair. To date, only one extracellular SOD (EC-SOD; SOD3), a Cu/Zn enzyme isolated from human placenta, has been identified and sequenced [9]. In common with these well-characterized enzymes, the apparent role of the SOD found in seminal fluid is to pre-empt the deleterious action of O_2^- by removing it before it can react to produce toxic by-products, such as lipid hydroperoxides. Although high levels of SOD activity have been reported in ejaculated semen [10], the source of this seminal SOD activity has not been definitively established. Indeed, it has been argued that

the observed levels in human semen could be accounted for by neutrophil contamination [11]. Given its importance, we have undertaken an investigation of seminal SOD at the molecular level. Here we describe the cDNA cloning of a novel rat epididymal SOD and the distribution of its corresponding transcript. We discuss the deduced primary structure of rat epididymal SOD in relation to that of the human EC-SOD and those of cytosolic SODs. The predicted characteristics of rat epididymal SOD suggest roles for the enzyme, and indeed O_2^- itself, in sperm maturation, storage and fertilization.

MATERIALS AND METHODS

Materials

Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, deoxynucleotides and *Sma*I-cut dephosphorylated pUC18 DNA were from Pharmacia, Milton Keynes, U.K. Hybond-N nylon membranes were obtained from Amersham International, Amersham, Bucks., U.K. [γ - ^{32}P]ATP (> 3000 Ci·mmol⁻¹) and random hexamer DNA labelling kits were from Du Pont-NEN, Stevenage, Herts., U.K. Nitrocellulose filters and micro-dialysis membranes were from Schleicher und Schüll and Millipore respectively. All other chemicals were of AnalaR grade or the purest grade available. Fresh tissue samples were from adult male Wistar rats (*Rattus norvegicus*) with the exception of fresh placental samples, which were from female rats at 16 days of gestation. Construction and storage of the rat epididymal cDNA library has been described previously [8].

RNA isolation and Northern-blot analysis

Castration and subsequent testosterone treatment of rats and isolation of RNA were as previously described [12]. Total RNA was subjected to Northern-blot analysis following fractionation

Abbreviations used: SOD, superoxide dismutase; EC-SOD, SOD3, extracellular superoxide dismutase; GPX, glutathione peroxidase; nn, nucleotides.

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The nucleotide sequence of rat (*Rattus norvegicus*) epididymal superoxide dismutase cDNA will appear in the EMBL Nucleotide Sequence Database under the accession number X68041.

by electrophoresis through a formaldehyde-containing 1.1% agarose gel. Following capillary transfer to a Hybond-N nylon membrane, samples were prehybridized and hybridized as described previously [13] using a cDNA insert labelled with ^{32}P to high specific radioactivity, employing a random-priming protocol [14]. Equivalent track loadings were verified by re-probing filters with mouse α -actin cDNA [15].

Isolation and sequence analysis of epididymal SOD cDNA

Approx. 5×10^4 rat epididymal cDNA clones were transferred to nitrocellulose filters and screened for those possessing cDNA inserts that hybridized to a 4-fold redundant oligonucleotide ($^5\text{CAGGTCCTCCGACTGGTGSACGTGGA}^3$) derived from nucleotides 407–432 (corresponding to amino acid residues 95–103) of human EC-SOD [9]; Figure 2 below). Hybridization was for 40 h under conditions of moderate stringency ($6 \times \text{SSC}$, 45°C) ($1 \times \text{SSC}$ is 0.15 M NaCl/0.015 M sodium citrate). After two further rounds of clone purification by re-screening under identical conditions, plasmid DNA from a single strongly hybridizing clone, prE-SOD, was isolated and its cDNA insert subjected to sequence analysis. Sequencing of prE-SOD was completed on both DNA strands using a custom primer walking strategy and employing a Du Pont Genesis 2000 automated sequencer. The prE-SOD cDNA sequence obtained in this way was compiled and aligned using the LASERGENE suite of programs (DNASTAR, West Ealing, London, U.K.).

PCR analysis of SOD transcripts

Total RNA (2 μg) or polyadenylated RNA (1 μg) was used to direct oligo-dT_{12–18}-primed cDNA synthesis by avian-myeloblastosis-virus reverse transcriptase. PCR was then carried out on one-tenth of this cDNA preparation in 10 mM Tris/HCl, pH 8.9, containing 50 mM KCl, 1.5 mM MgCl₂ and 100 $\mu\text{g} \cdot \text{ml}^{-1}$ gelatin, for 30 cycles using the following parameters for each cycle: 94°C for 1.5 min; cool to 58°C over 1.5 min; hold at 58°C for 2 min; rapidly heat to 72°C ; hold at 72°C for 2 min; rapidly heat to 94°C . Three combinations of overlapping primers {A, $^5\text{AGTTCGGGGACCTGAGCCAGGGCTG}^3$ [nucleotides (nn) 479–503; see Figure 1 below] and $^5\text{CAGGTCGTCCTCGC-CAGCGTGGAC}^3$ (nn 690–667); B, $^5\text{GGCTGCGAGTCCAC-CGGNCCNCA}^3$ (nn 499–521) and $^5\text{CAGGTCGTCCTCGCC-AGCGTGGAC}^3$ (nn 690–667); C, $^5\text{AGTTCGGGGACCTGA-GCCAGGGCTG}^3$ (nn 479–503) and $^5\text{CCCAGGTCGTCCTC-GCCNGCRTG}^3$ (nn 692–670)} were employed. These primers, which correspond to regions of sequence disparity between rat epididymal and cytosolic SODs, were designed to establish whether identical extracellular SOD transcripts were present in epididymis and placenta.

Measurement of SOD activity in testicular fluid and epididymal secretions

Luminal fluid was obtained from the cauda epididymidis of normal rats by retrograde injection of mineral oil via the vas deferens and collection of the extruded contents into a micro-pipette placed proximal to the site of injection. This procedure reduces contamination from blood and lymph to negligible levels. Caudal fluid was transferred to capillary tubes, centrifuged at 10000 g for 1 h at 4°C and supernatant plasma separated from the sperm pellet. Testicular secretions were collected by puncturing the rete testis of 18 h-efferent-duct-ligated rats and

centrifuged as described above. Sperm-free fluids were dialysed overnight against 1000 vol. of phosphate-buffered saline, the protein content measured [16], and either used directly or diluted further in phosphate-buffered saline. SOD activity was measured as described by Paoletti and Mocali [17] with purified bovine erythrocyte SOD as a positive control. In this assay system [17], 1 unit of SOD activity is defined as the amount of enzyme required to inhibit the rate of NADH oxidation of the control by 50%.

RESULTS AND DISCUSSION

Cloning and sequence analysis of rat epididymal SOD cDNA

After the screening of approx. 5×10^4 rat epididymal cDNA clones, a single strongly hybridizing clone, designated prE-SOD, was isolated and its cDNA insert sequenced. The prE-SOD cDNA insert (Figure 1) is apparently truncated with respect to its corresponding mRNA, as judged by the absence of an obvious polyadenylation/cleavage signal at its 3' end. However, comparison of its length (1244 nucleotides) with that of the complete transcript on Northern blots (1.56 kb; see Figure 3 below) suggests that it is almost full length after allowance for a poly (A) tail. The nucleotide sequence of prE-SOD cDNA possesses an open reading frame encoding a translational product of 244 amino acid residues, commencing with the 5'-proximal ATG codon (nn 109–111; Figure 1) and extending to a TAA termination codon (nn 841–843; Figure 1). This predicted protein sequence shows a high degree of conservation with the sequences of human Cu/Zn cytosolic and placental extracellular SODs, which exhibit 37 and 65% identity respectively (Figure 2). Many of the catalytically important residues in human Cu/Zn (cytosolic) SOD are conserved in the predicted prE-SOD mRNA translational product, including the ligands to Cu (H46, H48, H63 and H120) and Zn (H63, H71, H80 and D83) (residue numbers refer to the human cytosolic sequence; Figure 2). Detailed structural studies on human cytosolic SOD have shown that it possesses a hydrogen-bonding network at the rim of the active-site channel, which electrostatically facilitates O_2^- diffusion [18–20]. Although the residues that comprise this network (E132, E133, K136 and T137 in the human cytosolic enzyme; Figure 2) are not conserved in either human placental or rat epididymal SODs, the corresponding residues in the primary structures of both maintain, or increase electronegativity, consistent with undiminished predicted reaction rates [18]. Thus the predicted protein product of prE-SOD should retain many of the cardinal features of the Cu/Zn SODs.

The epididymis is a major site of extracellular SOD activity and prE-SOD mRNA

Sequence comparison with human EC-SOD (Figure 2) suggests that the putative rat epididymal enzyme is also secreted, since it not only exhibits extensive amino acid conservation (compared with either rat or human cytosolic SODs) but also is of a similar size. Moreover, both amino acid sequences possess analogous candidate N-terminal signal peptides. These findings strongly suggest that the rat epididymal SOD encoded by prE-SOD mRNA is also secreted. Further evidence that SOD is secreted by the epididymis is shown by direct measurements of enzyme activity in the luminal fluid. Reliable SOD activity could not be detected in rete testis fluid, but cauda epididymal plasma contained 235 ± 87 units/mg of protein, a value comparable with that found in liver cytosol, one of the richest sources of the enzyme [17].

To determine the tissue distribution of prE-SOD transcripts, RNA from rat liver, testis, placenta (at 16 days' gestation) and

1 666GAGGAGCTCAGAGGCTCTTTCTCAGGCTCTAGCTG66TCTGCTGTACTTCCAGAGGAAACGTTCTTGGGAGAGCTTGTCAAGTGTGGAACTCAGCC
 50 100 150
 Met Val Ala Phe Leu Phe Cys Asn Leu Leu Val Ala Cys Gly Ser Val Thr Trp Thr
 -18 -10 -1 +1
 200 250 300
 ATG TCA GAT ACC GGA GAG TCC GTC GAT TTA GCA GAC CCG CTT GAC CTG GTT GAG AAG ATA GGC GAC ACG CAC TCC AAA GAC CTG GAG ATC TGG ATG GAG CTA GGA AAA CAA CCG GAG GCG GAT GCC AGG GAG ATG CAC GCA
 Met Ser Asp Thr Gly Glu Ser Gly Val Asp Leu Ala Asp Arg Leu Asp Arg Leu Val Glu Lys Ile Gly Asp Thr His Ser Lys Asp Leu Glu Ile Trp Met Glu Leu Gly Lys Gln Arg Glu Ala Asp Ala Arg Glu Met His Ala
 10 20 30 40 50
 350 400 450
 GTC TGG AAG GTA CAG CCC TCA GGC ATG CTG CCT CCC GAT CAG CCA CAG ATC ACA GGC TTG GTC CTC TTC CCG CAG CTG GGG CCC AGC TCC AGA CTT GAG GCG TCC TTC CCA GCC GAG CAG AAC ACC TCC
 Val Cys Arg Val Gln Pro Ser Ala Met Leu Pro Asp Gln Pro Gln Ile Thr Gly Leu Val Leu Phe Arg Gln Leu Gly Pro Ser Ser Arg Leu Glu Ala Ser Phe Asn Leu Glu Gly Phe Pro Ala Glu Gln Asn Thr Ser
 60 70 80 90
 500 550 600
 AAC CAC GCC ATC CAC GTG CAT GAG TTC GGG GAC CTG AAG CAG GGC TGG GAG TGG ACC ACC GGA CCA CAC TAC AAC CCG CTG GGT GTG GCG CAC CCA CAG CAC CCG GGG GAC TTC GGC AAC TTC GTG GTG CCG GAT GGC CCG CTT TGG
 Asn His Ala Ile His Val His Glu Phe Gly Asp Leu Ser Gln Gly Cys Glu Ser Thr Gly Pro His Tyr Asn Pro Leu Gly Val Pro His Pro Gln His Phe Val Val Arg Asp Gly Arg Leu Trp
 100 110 120 130 140 150
 650 700 750
 AAG CAT CGA ATG GGC CTG GCG ACG TCA CTG GCG GGA CCG CAC TCG ATC TTG GGC CCG GCT GTG GTG GTC CAC GCT GGC GAG GAC GAC CTG GGT AAA GGT GGC AAC CAG GCG ACG GTG CAG AAC GGC AAC GCA GGT CCG CCG CTC
 Lys His Arg Met Gly Leu Ala Thr Ser Leu Ala Gly Thr His Ser Ile Leu Gly Arg Ala Val Val Val His Ala Gly Asp Asp Leu Gly Lys Gly Glu Asn Gln Ala Ser Val Gln Asn Gly Asn Ala Gly Arg Arg Leu
 160 170 180 190
 800 850 900
 GCC TGC TGC GTG GTA GGC ACC AGC AAC TCG GAG GCC TGG GAG ACG CAG ACA AAG GAG CCG AAG AAG TGG GAG AGC GAG TGC AAG ACC ACT TAA GCATCACCCAGGCGCCCTAGCCTAGCTGCTGTGTGTGCATAGATGCTCCACACAGCGCCT
 Ala Cys Cys Val Val Gly Thr Ser Asn Ser Glu Ala Trp Glu Ser Gln Thr Lys Lys Lys Trp Arg Trp Glu Ser Glu Cys Lys Thr Thr
 200 210 220 225 230
 950 1000 1050
 CTAGACGCTCCAGTCACTCAGAGGCTCTTGGGTGTCTAGACTGACGCTTCCAGAGACCTCAATGCGCTGTGTGCGCCCGCACACTCTTCCACATACCCCGAGACACCTCTGTATGGCTCAGATGCTTCAAGAACCTCTCTGCGCCACGTCACGAGCCCGAGATGTCCACAGTGCCTGCGCCACTGTT
 1100 1150 1200 1244
 CTCGAGACAGGAGACATTTTGTAACTAGGAATCCTTTACACACCCTATGCACTCCAGAGACCCAACTCCTTGTGCTAGGTCCACCTCGAACTACTTTATGCCCCAAGACAACTCCCATAGGCCCTAGCATGCCACACTTCCGC

Figure 1 Nucleotide and deduced amino acid sequences of rat epididymal secretory SOD (pr-SOD) cDNA

The underlined region (nn 504–666) was amplified by PCR using cDNA prepared from rat placental RNA and found to be identical with the epididymal sequence (see the text).

Figure 3 Identification of rat extracellular SOD transcripts by Northern-blot analysis

Total RNA (10 μ g/lane) was isolated from frozen rat tissues, electrophoresed on a denaturing agarose gel, blotted and probed with a rat epididymal SOD (prE-SOD) cDNA insert as described in the Materials and methods section. RNA samples were from liver (lane 1), 16-day placenta (lane b), testis (lane c), caput epididymidis (lane d), cauda epididymidis (lane e), total epididymis from 14-day castrated animals (lane f), total epididymis from 14-day castrated animals subsequently treated for 4 days with testosterone (lane g), and total epididymis from normal untreated animals (lane h). prE-SOD transcript size was calculated for its mobility relative to 28 S and 18 S rRNAs. The blot was subsequently reprobed with a cloned actin cDNA (results not shown) to confirm the integrity of RNA samples and to ensure equivalent lane loadings.

epididymis was subjected to Northern-blot analysis using prE-SOD cDNA as probe (Figure 3). Although the epididymis exhibited a relatively abundant hybridizing transcript of 1.56 kb (Figure 3), considerably lower steady-state levels of an identically sized hybridizing transcript were detected in the other tissues (Figure 3). Although these low levels of hybridizing mRNA cannot correspond to cytosolic SOD transcripts (which are considerably shorter), this finding does not preclude the possibility that identical EC-SOD transcripts are expressed in the placenta (among other tissues) and epididymis. This would imply that human EC-SOD is simply a species homologue of rat epididymal SOD.

In order to characterize further the relationship between rat placental and epididymal SODs, cDNA synthesized from total RNA isolated from placenta and epididymis was subjected to PCR using prE-SOD primers. Three different primer combinations were used (see the Materials and methods section), and each produced a major product of identical size with placental and epididymal cDNA. Furthermore, the cDNA sequences of the placental SOD PCR products were identical with the corresponding region of prE-SOD, strongly supporting the notion that placental and epididymal EC-SOD mRNAs (and presumably prE-SOD-hybridizing transcripts in the other tissues examined; Figure 3) are the same. However, of the tissues examined, the epididymis is clearly the major site of EC-SOD expression.

Hormonal regulation and localization of rat secretory SOD mRNA in the cauda epididymidis

Studies on the effect of androgen withdrawal on steady-state prE-SOD mRNAs levels in the epididymis revealed a decrease of approx. 4-fold by 14 days post castration, with a further slight decline 4 days after subsequent testosterone administration (Figure 3). This indicates that prE-SOD epididymal transcript levels are not as androgen-sensitive as several other characterized epididymal mRNAs (for example, see [8,12,13]), since parallel experiments with the same RNA samples using a different probe encoding an androgen-sensitive transcript (ESP 1; see [12]) resulted in re-induction after testosterone administration. Interestingly, more precise epididymal localization of prE-SOD

mRNA showed it to be concentrated in the cauda epididymidis and present at a much reduced level in the caput epididymidis (Figure 3). This finding may have physiological significance, given that spermatozoa can be stored in the cauda epididymidis (the more distal region of the epididymis) for days or weeks without losing their viability. Presumably prolonged storage in this way in a well vascularized (and oxygenated) tissue enhances the cumulative risk of peroxidative O_2^- -mediated aging. Spermatozoa themselves contain substantial amounts of SOD [5,10], but it is compartmentalized within the cell and would not be available to protect the external face of the plasma membrane. Secreted prE-SOD could assume such a protective role and, together with epididymal GPX, would form an important defence mechanism against free-radical damage to spermatozoa.

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