SUPEROXIDE DISMUTASE ACTIVITY OF ANTIBODIES PURIFIED FROM HUMAN ATHEROSCLEROTIC LESIONS

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ABSTRACT

The superoxide dismutase (SOD) family of enzymes is involved in the maintenance of oxidant/antioxidant balance in vascular tissue where it is expressed at high levels. In this report we show that there is 2-3 times more superoxide dismutase (SOD) activity in PBS extracts of lesioned arterial intima than normal intimal tissue. The source of this additional lesion-specific activity appears to be catalytic antibody molecules (IgG-SOD), which may be of importance during the atherosclerotic inflammatory response.

INTRODUCTION

Tissue damage caused by oxidizing free radicals is thought to contribute to ageing and avariety of diseases including atherosclerosis [1]. Tissues where high levels of oxygen radicals are produced exhibit high SOD activity and with the exception of rats and mice, arterial wall contains the highest SOD activity of all the tissues tested [2]. The presence of this high activity suggests that the control of superoxide levels is necessary for the normal functioning of these tissues. Since high SOD levels can initiate, as well as terminate lipid peroxidation [3] inadequate control of SOD activity within vascular tissue might alter the antioxidant capacity of vascular tissue, thus increasing the degree of oxidative modification of tissue components and therefore the risk of atherosclerosis.

Histological evidence for the accumulation of IgG molecules within the arterial wall of atherosclerotic lesions has been reported [4]. Since SOD activity of antibodies has recently been reported we wished to investigate whether this activity was associated with progression of the atherosclerotic lesion [5]. Unlike "classic" CuZnSOD antibodies exhibit an acidic pH maximum of pH 6.45 which is within the pH range thought to be associated with the atherosclerotic lesion [6].

In this report we measure the SOD activity of PBS and collagenase extracts of normal and lesion samples of human aortic intima. The results are discussed with regard to possible roles for IgG-SOD activity in atherosclerosis.

EXPERIMENTAL

- *Pathological specimens:* Human aortic specimens were procured from necropsy on 21 patients (age range 48 - 85 years, 16 male, 5 female) at Addenbrooke's Hospital. Outer media and adventitia were separated and discarded and lesions were classified on the basis of macroscopic appearance as either normal, fatty streak or advanced lesion. In some cases several lesion samples were available as well as a single sample of normal artery.

- *Extraction of aortic tissue:* Pieces of artery (approximately 100 mg wet weight) were briefly rinsed in phosphate-buffered saline (PBS), cut into approximately ten pieces, and placed in sterile 1.5 ml Eppendorf tubes. Samples were suspended in 1.0 ml of PBS, sonicated three times at 23 HZ with a 3mm needle probe for 3 seconds, then extracted by circular mixing for 20 min

and the final supernatant decanted. The procedure was repeated until no further protein could be detected by silver staining of extracts which had been separated by SDS-PAGE. Residual tissue (in 1.0 ml PBS) was delipidated with 10 ml diethyl ether and excess solvent was evaporated with a stream of argon. Washed tissue was digested overnight (16 h) at 37°C with 50 µg of Clostridial type VII-S bacterial collagenase (1800 Units/mg) and soluble protein was released from the washed tissue by sonication. Extracts were finally cleared of insoluble tissue fragments by centrifugation at 5000 g for 10 min on a bench top centrifuge and supernatants were stored at -80°C. Estimation of the protein content of extracts was determined by reaction with BCA reagent (Pierce).

- *Assay for SOD activity* : SOD activity of aortic extracts was measured on the basis of the rate of production of hydrogen peroxide (1.0 µmol H_2O_2/min = one Unit), detected by xylenol orange assay [7]. A second assay allowed SOD activity to be visualised on the basis of achromatic staining and required tissue extracts to be separated by electrophoresis on non-denaturing 5-12% acrylamide gels [8].

In order to determine which components of aortic extracts had SOD activity, IgG was immunoprecipitated from tissue extracts by addition of 50 µl of Staphylococcal protein A-bearing cells to 1 ml of tissue extracts. Samples were kept for 30 min with circular rotation at 4°C before protein A-antibody complexes were recovered by centrifugation at 3000 g. Pellets were resuspended, then washed three times with 1 ml aliquots of PBS prior to assay for SOD activity. Commercially available CuZn-SOD and IgG were used as positive controls for SOD and IgG-SOD activity and in order to determine whether IgG-SOD activity was dependent on Cu (II), the inhibitor of CuZn-SOD activity, diethyldithiocarbamic acid (DDC), was added to control mixtures to a final concentration of 10 µM.

RESULTS AND DISCUSSION

- *Extraction of protein*: PBS extracts of lesion samples had higher levels of scavenging activity on gels than extracts of normal tissue. Immunoprecipitation of IgG from lesion samples removed approximately 50% of this activity but had no effect on extracts of normal tissue. No further activity was removed by addition of a second aliquot of protein A and the activity lost from lesion extracts was fully recovered by assay of immunoprecipates following their resuspension in PBS.

Collagenase extracts of aortic intima produced a second pool of SOD activity which was unaffected by treatment with protein A. Western blot analysis of collagenase extracts confirmed that IgG molecules were not present in these samples. The SOD activity of both normal and lesion extracts was almost completely inhibited by DDC and like commercially available CuZnSOD and IgGs both were heat-labile, being completely inactivated by boiling for ten minutes. Protein A or albumin alone exhibited no scavenging activity when applied to gels at loadings of between 1-30 µg protein per lane.

Table 1. ***SOD activity of PBS and collagenase extracts of normal and lesion matched pairs from human aortic intima*** **A) PBS extracts of human aortic intima were assayed for SOD activity at pH 7.81 and 6.45 before and after treatment with either protein A or DDC. B) The remaining tissue was digested with collagenase and the soluble products released by this treatment were also assayed for SOD activity as described in Table 1A. See Experimental for further details.*NA=No adds.**

Disease status	SOD activity at pH 7.81 (µmol H202/µg protein/min)			SOD activity at pH 6.45 (µmol H202/µg protein/min)		
	NA*	+ DDC	+ protein A	NA	+ DDC	+ Protein A
Normal	6.61 +/- 1.34	2.54 +/- 0.87	5.20 +/- 1.88	3.87 +/- 0.61	0.87 +/- 0.13	4.45 +/- 0.18
Lesion	11.3 +/- 2.12	2.94 +/- 0.75	5.02 +/- 0.92	12.2 +/- 3.10	3.92 +/- 0.99	1.83 +/- 0.86

A

Disease status	SOD activity at pH 7.81 (µmol H202/µg protein/min)			SOD activity at pH 6.45 (µmol H202/µg protein/min)		
	NA	+ DDC	+ protein A	NA	+ DDC	+ Protein A
Normal	5.66 +/- 1.46	0.82 +/- 0.27	5.85 +/- 1.46	1.74 +/- 0.56	2.31 +/- 0.4	2.77 +/- 0.60
Lesion	4.03 +/- 1.25	0.44 +/- 0.16	3.06 +/- 0.50	2.41 +/- 0.73	1.52 +/- 0.3	2.24 +/- 0.64

B

When PBS extracts were assayed for SOD activity on the basis of their hydrogen peroxide-generating activity gels (Table 1.), the pattern of activity resembled that of their scavenging activity on gels. At pH 7.81 this activity was approximately twice as high in extracts of lesion (11.3 +/- 2.12 U/mg) than in the normal tissue (6.61 +/-1.34 U/mg), both activities being strongly inhibited by DDC to give 2.94 +/- 0.75 and 2.54 U/mg respectively. Incubation of PBS extracts with protein A removed approximately half of the total lesion extracts activity but had little or no effect on extracts of normal intima. A reduction of the assay pH to 6.45 had little or no effect on the activity of the lesion extracts, but approximately halved the activity of normal tissue extracts, consistent with a proportion of the lesion antibodies having a low pH maximum for IgG-SOD activity.

Assay of collagenase extracts at pH 7.81 showed that the activity of normal and lesion extracts was comparable (5.66 +/- 1.46 and 4.03 +/- 1.25 U/µg protein respectively), and that they were both inhibited by between 50-70% with DDC. Reduction of the pH to 6.45 approximately halved the activity of normal and lesion extracts to 1.74 +/- 0.56 and 2.41 +/- 0.73 U/µg protein respectively. As with the scavenging assay no H_2O_2 generating activity was removed from either normal or lesion matched pairs by protein A treatment.

A significant correlation was found between severity of lesion progression and SOD activity in PBS extracts of human aortic intima. Assay of PBS extracts at pH 7.81 showed that there was 3.96 +/- 0.01 U/µg protein in normal artery (n=11), 6.32 +/- 0.59 U/µg protein in fatty streaks (n=11) and 13.7 +/- 1.21 U/µg protein (n=9) in advanced lesion (comparing disea-

sed tissue with normal tissue gave p values of < 0.001). A similar pattern of activity was calculated for data obtained for SOD assay at pH 6.45, with activity increasing with severity of lesion from 1.91 +/- 0.30 U/µg protein in normal artery to 4.1 +/- 0.71 U/µg protein in fatty streaks and 13.2 +/- 1.31 U/µg protein in advanced lesions, (again p < 0.001). The PBS-extractable activity of highly advanced lesions, which were capped and filled with gruel, did not however follow the general trend of increasing with severity of disease, since they contained only 5.05 +/- 0.94 U/µg protein (n=10).

Currently, the structure/function relationships which govern IgG-SOD activity are poorly understood, but antibody molecules are known to have a structural similarity to SOD [9], and the observation that IgG-SOD activity was inhibited by DDC suggests that bound Cu (II) ions may contribute to the enzymic property of lesion antibodies. These molecules appear to provide a means of delivery for SOD activity to sites of arterial inflammation and therefore may contribute to the mechanisms which control the progression of atherosclerosis.

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