From the Center for Reproduction of Endangered Species,
Zoological Society of San Diego

6544

RED BLOOD CELL ENZYMES IN THE BLACK RHINOCEROS (*DICEROS BICORNIS*): INITIAL MOLECULAR STUDIES OF CATALASE

By M. B. W o r l e y and Ann C. C a r p e n t e r

Introduction

In recent years an increased emphasis has been placed on captive breeding programs and intensely managed *in situ* populations as components of an international conservation effort for the black rhinoceros. As a result, there has been an evolving awareness of the diseases that affect this species (MILLER, 1993; KOCK and GARNIER, 1993). Severe anemia as a result of acute episodic hemolysis has been observed in a number of zoological parks (MILLER and BOEVER, 1982) and is the leading cause of death among captive-raised black rhinoceroses (KOCK and GARNIER 1993; PAGLIA and MILLER, 1993). This disease has not been documented in the white rhinoceros (*Ceratotherium simum*) or the Asian rhinoceroses.

Extensive investigations concerning the possible causative factors of the hemolysis have revealed that the black rhinoceros possesses a unique enzyme and metabolic profile in normal erythrocytes compared to humans and other mammals (PAGLIA, 1993). The activity of a number of cellular enzymes that play a role in defense against oxidative insult are markedly decreased compared to all other known species (PAGLIA et al.,1986). Catalase deficiency has been proposed to be a species-wide characteristic of black rhinoceroses in which erythrocyte enzyme levels are only 2-3% of that in human erthrocytes (PAGLIA and MILLER, 1992; PAGLIA, 1993). Because white rhinoceroses are not susceptible to hemolysis and share nearly all the red cell metabolic characteristics of black rhinoceroses, with the exception of the catalase deficiency, it is possible catalase plays a pivotal role in the pathogenesis of acute episodic hemolysis in the black rhino.

The molecular bases for acatalasemia in humans and mice have recently been elucidated (KISHIMOTO et al., 1992; SHAFFER and PRESTON, 1990; WEN et al., 1990). Similar findings for catalase of the black rhinoceros could potentially establish screening procedures to identify individuals at high risk for developing hemolytic disease. This report describes the initial molecular cloning and sequencing of the black rhino catalase coding region and notes some interesting features of the translated amino acid sequence relative to the enzyme sequence in other mammals.

Materials and methods

Construction and screening of cDNA library.

Primary black rhinoceros fibroblasts, grown and expanded from a skin biopsy taken from an animal that has never experienced a hemolytic episode, were provided by Marlys H o u c k, Center for the Reproduction of Endangered Species. Rhinoceroses rarely produce reticulocytes and the reported DNA sequences of catalase from different tissues are nearly identical. Total cytoplasmic RNA was isolated from cultured fibroblasts (CHOMCZYNSKI and SACCHI, 1987) and double stranded cDNA was synthesized from poly (A)+ messenger RNA. The cDNA was blunt end ligated to Eco RI linkers and packaged in the lambda phage vector, lambda gt10 (Stratagene, Inc., La Jolla, California, USA). The amplified library was screened for catalase sequences using a random primed phosphorus-32-deoxyadenosine triphosphate (^{32}P-dATP)-labeled human pCAT10 cDNA as a probe (American Type Culture Collection, Rockville, Maryland, USA). Hybridization was overnight at 42°C and membranes were washed successively at 42°C, twice for 5 min each in 2X SSC plus 0.1% SDS and twice for 30 min each in 1X ssc plus 0.1% SDS. The membranes were air-dried and exposed to X-ray film. The screening was repeated to plaque purify positive clones.

Subcloning

DNA was isolated from lambda phage plate lysates and digested with Eco RI. Electrophoresis in 1% agarose gels was used to assess insert size. DNA containing an approximately 1.8 kb insert was cut from the gel, electroeluted from the agarose slice, phenol/chloroform extracted and ethanol precipitated. Resuspended DNA was made blunt-ended with the Klenow fragment of *E. coli* DNA polymerase 1 and ligated into the Sma1 site of phagemid Bluescript II (SK-) (Stratagene, Inc., La Jolla, California, USA). Transformation-competent *DH5 E.coli* cells were prepared and transformed with ligated DNA. Transformants were plated in agar with X-gal. White colonies were picked and grown in liquid culture and DNA was isolated in preparation for sequence analysis.

Sequencing

Preliminary sequencing by the dideoxy chain terminating method (SANGAR et al., 1977), was performed using primers corresponding to the sequence of the T3 and T7 promoters flanking the multiple cloning site of Bluescript. Sequencing was completed with synthetic oligonucleotide primers designed to extend the sequences obtained with the previous primers. Both strands were sequenced to obtain a consensus.

Computer analysis

Nucleotide sequences were aligned, edited, and translated using the MacVector and AssemblyLIGN software programs (Eastman Kodak Co., New Haven, Connecticut, USA). Black rhinoceros catalase nucleotide and amino acid sequences were compared with known catalase sequences of other mammals using the Entrez document retrieval system (National Center for Biotechnology Information, National Library of Medicine, Bethesda, Maryland, USA).

Results

Hybridization screening of the black rhinoceros fibroblast cDNA library with the human catalase pCAT10 cDNA probe resulted in the detection of one clone. Three individual clones from the third round of plaque purification were selected for sequencing to establish a consensus sequence. All three clones, which were 1,743 basepairs (bp) in length, possessed an identical sequence. Comparison of the sequence of one of these clones, designated pBRCAT3.1, with human catalase sequence revealed that pBRCAT3.1 contained 1,369 bp of the coding region and 377 bp of the 3' untranslated region of the catalase gene. The rhinoceros clone is missing the 5' untranslated region and 216 bp of the beginning of the coding region. It contains 86% of the coding region of the black rhinoceros catalase gene.
Comparison of the translated amino acid sequence of pBRCAT3.1 with known amino acid sequences of several mammalian catalases revealed a high degree of homology as seen in Table 1. Percent homology ranged from a high of 95.2% with bovine catalase to a low of 90.9% with rat catalase. Although the amino acid substitutions were dispersed throughout the coding region, 10 substitutions between rhinoceros and human catalase occurred in the first half of the sequence and 33 substitutions occurred in the second half.

Table 1: Homology of catalase amino acid sequences. Comparisson of percent homology of amino acid sequence of pBRCAT3.1 and several mammalian catalase sequences

	B.RHINO	COW	HUMAN	MOUSE	RAT
B.RHINO	100	95.2	92.3	92.1	90.9
COW	95.2	100	94.3	94.5	92.9
HUMAN	92.3	94.3	100	91.9	90.5
MOUSE	92.1	94.5	91.9	100	96.0
RAT	90.9	92.9	90.5	96.0	100

A hypervariable region encompassing 16 amino acids and represented by residues 350 through 365 of pBRCAT3.1 was found in all five of catalase sequences as seen in Table 2. When compared to the human, bovine, mouse, and rat sequences for this region, pBRCAT3.1 differed by 10, 7, 7 and 6 amino acid substitutions, respectively. Therefore, although rhinoceros and human catalase sequences are 92.3%

homologous through the entire translated region of pBRCAT3.1, they are only 37.5% homologous in the 16 residue hypervariable region.

Table 2: Amino acid sequences of hypervariable region. Comparative amino acid sequences of a hypervariable region corresponding in position to residues 350 through 365 of pBRCAT3.1. Designations for the triple-letter codes are: alanine (Ala), arginie (Arg), asparagine (Asn), aspartic acid (Asp), cysteine (Cys), glutamic acid (Glu), glutamine (Gln), glycine (Gly), histidine (His), isoleucine (Ile), lysine (Lys), phenylalanine (phe), proline (Pro), serine (Ser), threonine (Thr), tyrosine (Tyr) and valine (Val)

B.RHINO	Lys	Phe	Arg	Cys	Ser	Pro	Asp	Val	Gln	Arg	Phe	Asn	Ser	Ala	Ser	Glu
HUMAN	Ser	Ile	Gln	Tyr	Ser	Gly	Glu	Val	Arg	Arg	Phe	Asn	Thr	Ala	Asn	Asp
COW	Arg	Thr	His	Phe	Ser	Gly	Asp	Val	Gln	Arg	Phe	Asn	Ser	Ala	Asn	Asp
MOUSE	Ser	Val	Gln	Cys	Ala	Val	Asp	Val	Lys	Arg	Phe	Asn	Ser	Ala	Asn	Glu
RAT	His	Ser	Gln	Cys	Ser	Ala	Asp	Val	Lys	Arg	Phe	Asn	Ser	Ala	Asn	Glu

Discussion

The erythrocyte is the most susceptible cell in the mammalian body to oxidant stress (OGATA, 1991). Catalase plays a central role in oxidant defense and it has been estimated that over 99% of added H_2O_2 which enters the erythrocyte is destroyed by catalase, thereby supplying almost perfect protection to hemoglobin against oxidation by H_2O_2 (NICHOLLS, 1965). The markedly reduced catalase activity in black rhinoceros erythrocytes would almost certainly compound the restricted antioxidant capacity already inherently present (PAGLIA, 1993). The molecular bases for acatalasemia in Japanese patients (KISHIMOTO et al., 1992; WEN et al., 1990) and a specific strain of mouse (SHAFFER and PRESTON, 1990) have recently been determined. In human patients, a single nucleotide substitution severely limits the correct splicing of the gene RNA product resulting in a deficiency of catalase mRNA and, therefore, the enzyme product (WEN et al., 1990). In acatalasemic mice, a single base substitution resulting in a glutamine hisidine substitution may render the enzyme unstable under physiological conditions.

It has been suggested that catalase deficiency is a species-specific trait of the black rhinoceros. If so, it will then be difficult to determine differences between animals that have or have not experienced a hemolytic episode. A change in the chemical nature of the 16 residue hypervariable region (eg. becoming more or less hydrophobic) as a result of any one of the 10 substitutions between rhinoceros and human sequence might decrease the availability of specific sites to substrates, thereby diminishing the catalytic efficiency of the enzyme. This scenario might explain the diminished enzyme activity of rhinoceros erythrocytes compared to that of humans but it probably would not establish the difference between clinically affected and unaffected individuals.

Currently available molecular techniques and the data presented in this report can aid in answering two questions:

1) are there differences between the DNA sequence and possibly amino acid sequences of black rhinoceros and white rhinoceros catalases? and

2) are there minor sequence differences, as subtle as a single nucleotide base substitution, between the catalase coding regions of individual black rhinoceroses that can explain susceptibility to hemolysis?

Hopefully, data from experiments designed to answer these questions will lead to a better understanding of disease susceptibility that can be applied to improved management procedures for the conservation of this species.

Summary

Red blood cell enzymes in the black rhinoceros (Diceros bicornis): initial molecular studies of catalase

The enzyme catalase may play a pivotal role in the pathogenesis of acute episodic hemolysis in the black rhinoceros. We constructed a black rhinoceros fibroblast cDNA library and screened for catalase sequences with a human catalase cDNA probe. The DNA sequence of a positive clone that represented 86% of the coding

region of the catalase gene was determined. The translated amino acid sequence was 90-95% homologous to bovine, human, mouse and rat catalase sequences. However, a hypervariable region of 16 amino acids was only 37.5% identical between black rhinoceros and human sequences. This result may offer a possible explaination for the finding that catalase activity in black rhinoceros erythrocytes is only 2-3% of that found in human erythrocytes.

Zusammenfassung

Enzyme der roten Blutkörperchen beim Spitzmaulnashorn (Diceros bicornis): Erste Molekularuntersuchungen zur Katalase

Das Enzym Katalase könnte beim Spitzmaulnashorn in der Pathogenese der akuten, schubartig auftretenden Hämolyse eine entscheidene Rolle spielen. Wir erstellten eine cDNA-Karte der Fibroblasten für das Spitzmaulnashorn und überprüften diese auf Katalasesequenzen, wobei eine cDNA-Sonde für die Katalase beim Menschen verwendet wurde. Bestimmt wurde die DNA-Sequenz eines positiven Klons, die 86 Prozent des kodierenden Bereiches des Katalase-Gens repräsentierte. Die transkribierte Aminosäuresequenz entsprach zu 90 bis 95 Prozent den Katalasesequenzen beim Rind und Menschen sowie bei der Maus und Ratte. Ein hypervariabler Bereich von 16 Aminosäuren des Spitzmaulnashorns war jedoch nur zu 37,5 Prozent mit der entsprechenden Sequenz beim Menschen identisch. Dieses Ergebnis könnte möglicherweise eine Erklärung dafür bieten, daß bei anderen Befunden die Katalase-Aktivität der Erythrozyten beim Spitzmaulnashorn nur zwei bis drei Prozent der Aktivität in den Erythrozyten des Menschen ausmachte.

Résumé

Les enzymes des globules rouges chez le rhinocéros noir (Diceros bicornis): premières études moléculaires de la catalase

L'enzyme de catalase pourrait effectivement jouer un rôle central pour la pathogenèse de l'hémolyse aiguë et épisodique du rhinocéros noir. Nous avons élaboré une série d'ADNc de fibroblastes du rhinocéros noir que nous avons examinés au moyen d'une sonde de catalase ADNc humaine pour obtenir les valeurs de catalase. Nous avons déterminé la séquence ADN d'un clône positif qui a représenté 86 % de la région codante du gène de la catalase. La séquence d'amino-acides transcrite était homologue à 90 - 95 % par rapport aux séquences de catalase chez le bovin, l'homme, la souris et le rat. Pourtant, une région hypervariable de 16 amino-acides du rhinocéros noir n'était identique qu'à 37.5 % à la séquence humaine. Ce résultat pourrait expliquer la conclusion selon laquelle l'activité de la catalase des érythrocytes du rhinocéros noir représente seulement 2 - 3 % de celle des érythrocytes humains.

References

CHOMCZYNSKI, P. and N. SACCHI (1987):
Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analyt. Biochem. 162, 156-159.

KISHIMOTO, Y., MURAKAMI, Y., HAYASHI, K., TAKAHARA, S., SUGIMURA, T. and T. SEKIYA (1992):
Detection of a common mutation of the catalase gene in Japanese acatalasemic patients. Hum Genet. 88, 487-490.

KOCK, R.A. and J. GARNIER (1993):
Veterinary management of three species of rhinoceroses in zoological collections. In Rhinoceros Biology and Conservation. Zoological Society of San Diego. pp. 325-345.

MILLER, R.E. and W.J. BOEVER (1982):
Fatal hemolytic anemia in the black rhinoceros: case report and a survey. J. Am. Vet. Med. Assoc. 181, 1228-1231.

MILLER, R.E. (1993):
Health concerns and veterinary research in the North American black rhinoceros (Diceros bicornis) population. In Rhinoceros Biology and Conservation. Zoological Society of San Diego. pp.302-306.

NICHOLLS, P. (1965):
Activity of catalase in the red cell. Biochim. Biophys. Acta 99, 286-293.

OGATA, M. (1991):
Acatalasemia: a review article. Hum Genet. 86, 331-340.

PAGLIA, D.E. (1993):
Acute episodic hemolysis in the African black rhinoceros as an analogue of human glucose-6-phosphate dehydrogenase deficiency. Am. J. Hematol. 42, 36-45.

PAGLIA, D.E. and R.E. MILLER (1993):
Erythrocytes of the black rhinoceros (Diceros bicornis): Susceptibility to oxidant-induced haemolysis. Int. Zoo Yb. 32, 20-27.

PAGLIA, D.E. and R.E. MILLER (1992):
Erythrocyte ATP deficiency and acatalasemia in the black rhinoceros (Diceros bicornis) and their pathogenic roles in acute episodic hemolysis and mucocutaneous ulcerations. Proceedings of the American Association of Zoo Veterinarians. pp. 217-219.

PAGLIA, D.E., VALENTINE, W.N., MILLER, R.E., NAKATANI, M. AND R. BROCKWAY (1986):
Acute intravascular hemolysis in the black rhinoceros: erythrocyte enzymes and metabolic intermediates. Am. J. Vet. Res. 47, 1321-1325.

SANGER, F., NICKLEN, S. and A.R. COULSON (1977):
DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences, United States of America 74, 5463-5467.

SHAFFER, J.B. andK.E. PRESTON (1990):
Molecular analysis of an acatalasemic mutant. Biochem. Biophys. Res. Comm. 173, 1043-1050.

WEN, J-K., OSUMI, T., HASHIMOTO, T. and M. OGATA (1990): Molecular analysis of human acatalasemia: identification of a splicing mutation. J. Mol. Biol. 211, 383-393.

Address of authors: Michael B. W o r l e y
Center for Reproduction of Endangered Species
Zoological Society of San Diego
P.O. Box 551
San Diego, California 92112-0551, USA