DNA CORRELATES FOR ENZYME DEFICIENCIES IN BLACK RHINOCEROS

Mike Worley

TAPE 9A 584

Worley: About a year ago I talked to Don [Paglia] on the phone for 585 work and from Eric [Miller] and Evan [Blumer] and was interested in talking with them about some of the intramolecular mechanisms of these enzyme deficiencies. The reason I was interested was because at that time we were doing a lot of work with rhinoceros *major* histocompatability complex genes. We had constructed some DNA libraries called cDNA libraries from which we could extract the coding reagents of these genes that deliver genetic variation in the gene complex. We felt that these might be a good source in which to look at some of these enzymes, some of the genes associated with these enzymes. Don [Paglia] said, that sounds really great probably not very many people have a black rhinoceros cDNA library. We made a commitment to the at our laboratory in San Diego, and in the last year we have pursued that. I will present very briefly up to about a week ago some of the data we have.

This overview kind of overlaps what Don [Paglia] was talking about. There is a number of enzyme deficiencies in humans associated with hemolytic anemia. This is probably just a partial list of where these different loci are located on certain chromosomes. It is interesting that with the ADA excess you can get hemolytic anemia. Many of these are enzymes that do not mention what is there.

Jessup: All those syndromes are associated with different types of hemolytic anemia in humans?

Worley: The deficiency of each one of those enzymes with the exception of ADA is associated with hemolytic anemia.

Paglia: Yes, the deficiency of ADA is not. The ADA in hyperactivity is. That is because the picture I just showed you of the rhino being able to take adenosine into the ATP pool... When you have an ADA excess, it deaminates adenosine to inosine and that removes its availability of substrate to go back into the pool. So having hyperactive ADA deprives the red cell of an essential *cellic* pathway. So we have a paradoxical situation where increased activity of an enzyme wipes out an essential substrate and you end up with a hemolytic process. Some of these others are questionable. They are not all cause and effect. Adenylate kinase, *Ernie Boyd* had a case of two siblings with virtually no detectable adenylate kinase. One had the deficiency and the other one seemed quite normal.

Jessup: So they may have other genetic anomalies, other than these deficiencies that would account for their *anemic problems.*

Paglia: They have looked and looked and looked, but it is not clear. There are five families with that adenylate kinase deficiency, one of which is *Ernie's* family. You can say it is associated, but to say it is cause and effect is another matter. There is an association.

Jessup: I am just trying to get that clear, whether it is associated or clearly.

Paglia: In most instances it is pretty clear. Among these, glutathione peroxidase is questionable, AMPdeaminase is questionable. Is the mutase questionable still?

Smith: Glutathione reductase is questionable isn't it? It is real dependent on the *vitamin,* on the *flavin* intake

Paglia: The *flavin* associated enzyme. So most of them are pretty clear cut. G-6-PD, glucose phosphate, triosephosphate isomerase, hexokinase, pyruvate kinase, there is know question about those. But a lot of these have been reported in just a couple of families. It is associated, but proving cause and effect takes a lot.

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Worley: Some of the potential causes in those that are well established... Certainly the second one, amino acid substitution as a result of 001 mutation can lead to a change in the thermostability of the enzyme. I am not aware of the first one with some of the erythroenzymopathies, whether there is documented reduced level of mRNA, which has been transcribed. Certainly this is the case with other diseases. Take a look at 004 T-cell growth factor. There are some individuals in which there have been reduced levels of mRNA transcribed from the 006 gene. This condition leads to amino deficiency and has been classified as the primary immunodeficiency by the 007.

Here are some examples of amino acid substitution, although they are like 009 I guess it is by association. The residue number and where there is a substitution, are these four examples. The last three being more positively established. Because G-6-PD at this time is probably 40 plus mutations globally that lead to hemolytic anemia. In some of those patients in which it is a chronic problem, there has been talk of gene replacement therapy. Pyruvate kinase... These are just three substitutions that have been associated with hemolytic anemia. Just various changes... This 017 is a hydrophilic hydophobic amino acid that leads to conformation changes that again affect the thermostability of the enzyme. Just a change in the single methalene group in this residue causes problems.

So what we wanted to do is look at the rhinoceros. We had the cDNA libraries, we obtained cDNA probes for some of these enzymes, human origin probes. We wanted to see if we could fish some of these out. We began to obtain data that would let us quantitate levels of expression of these genes, as well as compare amino acid sequence of the end product between black and white rhino. Just a generalization that some or all of you may be aware of, is when you have a gene not all of the DNA in that gene is transcribed into RNA. Only some of it, generally called exons are transcribed into an RNA which is a coding region which is subsequently translated into the protein product. You can from this RNA, through a process of reverse transcription, make cDNA. By reverse transcriptase *it is prepared from* retroviruses. It is commercially available and will allow you to make cDNA libraries.

This is what we did. The technique is kind of outlined here. We utilized frozen vials of primary fibroblast cultures from what we call the "Frozen Zoo" at San Diego. These were taken from skin biopsies

and had been, in addition to karyotypic analysis of a number of species, very important in some of our work in my lab. We essentially take the primary fibroblast culture cells, extract RNA, select for mRNA, reverse transcribe that into cDNA, and then size select that and insert it into a vector. These are straight forward techniques. They are very routine. Then taking that vector containing that insert DNA, transform *E. coli* cells, plate them out, and then screen them for positive colonies with plaques with the appropriate probe; with most emphasis on catalase, using a human cDNA probe for catalase. We then find positive... Using a radioactive probe we expose these membranes to X Ray film. With a spatula we take them out of the auger, we then 046 out the DNA. We go through several passages of *black* purification, such that our petri dish at the end is essentially pure for each one of those clones. We then go ahead and sequence it.

What we found when we looked at our positive plaques, taking into account the number of positive plaques we found, the number we found in our plaques we put in to screen as that with same human cDNA probe we found approximately 35 times as many positive plaques in our white rhino cDNA library as in our black rhino cDNA library. This is not quantitative, it is very crude. But it suggests that in those two respective primary fibroblast lines there may be significantly more mRNA that have been transcribed and expressed for catalase in the white rhino, than in the black rhino. That remains to be seen. I will describe how we will go ahead and try to prove that.

Munson: Do all tissues make it evenly?

Worley: We do not know.

Munson: Would fibroblasts be one of the main catalase producers?

Worley: No, not in humans, very low levels of catalase is my understanding from the reading, red cell is very high.

Munson: And it is the same cell type with the white and black rhino, they are both skin fibroblasts as your source.

Worley: Yes, the same anatomic location, routine. We then went ahead and did some sequencing. What we do basically is... If you get full length cDNA clones, what you usually get is a full coding region, which is between to two bars here. You get just a little bit of sequence on the 5' end which is untranslated and you get what we call a poly-A tail, a bunch of A's, adenosines, adenines on the 3' end. So we are sequencing in from the ends. So what we have done is we have sequenced in from the 5' end and from the 3' end. We are still in the translated region in the 3' end. So what I am going to show you is our data from the left side or the 5' end. We have sequenced at this point about 21% of the entire coding region of the black rhino and the white rhino catalase genes. That is 333 bases and 111 amino acids. So what we find is that between human and white rhino there is 100% homology. If you know that an amino acid like leucine can have six different types of codons, in other words, I forget what it is for leucine, but it can be lets say CAT, CAG, GAT; so you can have base differences and still code for leucine. There were no nucleotide differences in that 333 bases between white rhino and human catalase. There was 100% homology of

nucleotide and amino acid sequence. The black rhino was 95.5%, which seems very close; and indeed I would guess that difference does not explain the potential difference in the number of positive plaques we found between the two libraries. I would say that is just not an acceptable explanation. That is still almost 96% homologous. Each one of those amino acid differences was due to one nucleotide change. So the nucleotide and amino acid sequences between the human and the black rhino is still 95.5%. I find that very interesting. Even though it is only 21% of the entire coding region of that gene, there is 100% homology between human and white rhino; and black rhino which is still 95.5%, to me that is a lot. That 5.5% difference is a lot. Why over time that has been different, I do not have an explanation. In those 111 amino acids, five substitutions are just here and we have not analyzed that out on a computer, where we have gone from a charge down from a hydrophilic to a hydrophobic, quite conformational changes. That might explain why there is a deficiency of the enzyme in the black rhino. It could be quantitative. Again, there may be less mRNA transcribed from the gene. That may be a simple explanation. Maybe something can be done about that.

What we will be in the process of doing when I get back, is continuing to sequence both directions. We will sequence both strands, get a consensus, and we will look at the entire amino acid sequence of the product. There is no reason to suspect that it will be any different. I think when you get towards the 3' end of the cDNA, or *carboxyterminous* of the protein, there may be a little more polymorphism. It will be interesting to see. What has come out of this of course, is not only a better understanding of the structure of catalase of rhinoceros, but now we have these cDNA probes. What we want to do is go back and look at different tissues. Don [Paglia] wants to look at actual enzyme levels. I want to look at levels of mRNA expression among the various tissues. This is primary fibroblasts from skin biopsy. Of course we would like to look at liver. We wish rhinos would make reticulocytes. We would like to make a reticulocyte cDNA library. I think that would be very helpful with some of the other enzymes.

This is where we are right now. I think it is significant. I really do not know what it means. I think we have a minimum of two different explanations or possibilities as to why there may be a catalase deficiency in black rhinos. We have also screened both the back and white rhino cDNA libraries with cDNA probes for glutathione-S-transferase. We got a number of clones out of both of those libraries. We have sequenced the ends of both of those. Whereas catalase seems to probably come from one gene family, there is a number of isoenzymes, GST. *We have not sorted* out what is going on with that yet.

Jessup: So what you are saying, is that there is some slight structural differences in the coding of the RNA, that results in some structural differences in catalase.

Worley: Some differences in the amino acid sequence in the black rhino in the first 21%.

Jessup: So it might not be really a deficiency of catalase, it might be a slightly difference in the structural shape, and thus the function of catalase.

Worley: It could be. I will not call these mutations like I showed for the human, because these are black rhino. You can not look at the amino acid sequence of back rhino catalase and it looks different from white rhino catalase, and say that those are mutations. Just for some reason they have evolved.

Smith: You can have a protein, but just not the active *vessel*. This is at the nucleotide level, this 100%, or at the amino acid level?

Worley: For the human and white rhino, both.

Paglia: So at the nucleotide level it is the same.

Worley: The first 21% for the tri-128. I was worried, but we knew it was the white rhino cDNA library. We probed *it for MHC*. It is not human! Catalase is a highly conserved gene.

Smith: There are some big changes 132. I mean histadine to glutamine and proline to alanine, those are some pretty big jumps. Hasn't catalase been crystallized and all that stuff?

Worley: Yes, I do not know if it is from human or rat, probably rat my guess would be. But I have not made a comparison in that first, that one fifth 5' end. But if you look at the whole sequence, if this carries on, I hope it is not 100% across the way with white rhino. I would be a little worried about that! Certainly human and white rhino here, are more homologous than human and rat, or human and bovine catalase.

Jessup: How many animals *are the libraries made from*, or one animal?

Worley: The libraries are made from one animal. What we can do now though, is make primers *through PCR* from RNA. We have got primary fibroblast cultures from I do not know how many individuals that have been given to me and we have grown up and frozen them down. So we have an array, a number of animals from which we can now go back and compare. Some have had hemolytic episodes, some have had mucocutaneous disorders.

Paglia: Did you see any dinucleotide *repeats* 147?

Worley: We are still in the *untranslated* region of the sequence, so we have...

Paglia: Down there the dinucleotide repeat sometimes occur down...

Worley: We have got a lot of A's, a lot of T's, with nothing consistent.

Bolin: That 100% correlation is frightening on the data on contamination of cDNA libraries, which I am sure you are aware of. But is there precedence for two widely divergent species having 100% match of gene?

Worley: But this is only the first 21%.

Bolin: So, hopefully it is not going to turn out that way. Ninety six is a lot of homology, that is pretty close.

Worley: We do not have, unless we slough something off from our hands, we have no human cell lines in my lab, and no 157. Certainly, the *MHC* that we got out of this library is non-human, it is rhinoceros.

Bolin: That means there is at least some rhinoceros DNA, it does not mean all. Do we have other correlates in which human *IO-1* and bovine *IO-1* are the gene sequence and are identical?

Worley: In segments of the coding regions of those genes, yes. Not along the entire coding regions, right.

Jessup: Are there not some genes that translate all the way from literally frogs to horses have tremendous 164.

Bolin: But not 100%. Proteins yes, not genes.

Munson: Close to it, like 99%, like the cofactors and stuff like that.

R. Kock: Perhaps I am going to speak out of term, maybe Evan [Blumer] is going to say something. With all this I keep hearing terms coming out like catalase deficiency and all this sort of business. I mean the black rhino is doing fine in very varied habitats where it has fantastically divergent feed. Its health problems and so on are not significant. I just feel we should be a little careful not to say there is something wrong with this animal. I think what we are doing is pushing this animal through some extraordinary circumstances. All this work needs to sort of focus on what we can do to avoid those circumstances, what conditions we can improve in the management of the animal, whether it be in the wild situation where it is confronted by different 173 and so on. And I really appreciate what this has done, but honestly I am a little worried it is going to get a bad reputation.

Worley: I appreciate what you are saying, and certainly what Nancy [Kock] said yesterday, that the major problem is poaching; but I also have to hear what Pete Morkel said. Even though, he said a lot of these problems need low tech answers, I also hear him saying that he thinks Namibia five years ago only had half the number of rhinos they do now. So just having the honor and pleasures of spending some time with Mike Kock and *Mark Atkinson* in the last three weeks in Zimbabwe. You talk about like Pete [Morkel] said, an intense management of rhinos. I think when you talk about intense management of animals, hopefully not to the point where they are in zoos, but if you look at some of these species in zoos, what I can tell you is that they are being exposed to things that they would not be naturally. I think that some of us need to think about those. I am interested in looking at the molecular basis of some of these problems. I think if we understand the mechanisms and molecular basis for somethings, then we can maybe turn around and apply some solutions. But I appreciate very much what you are saying, absolutely.

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