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Synthesis of metallothionein in the retina of cynomolgus monkeys

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Metallothionein cDNA was detected in the retinas of cynomolgus monkeys (*Macaca fascicularis*) using RT-PCR. Sequencing of this cDNA showed almost a hundred percent sequence similarity with metallothionein cDNA from rhesus monkey kidneys. There was only a single base substitution (ACC → ACU) for Thr 14. A comparison of the amino acid sequence also showed a high percentage similarity with metallothionein class II isolated from other sources. Previous reports of several laboratories have implicated the induction of metallothionein synthesis as a response to oxidative stress which in turn is due to solar bombardment and phagocytosis of photoreceptors, particularly rod outer segments, by retinal pigment epithelium.

Key Words: metallothionein, cynomolgus monkeys, retina, oxidative stress

THE EYE IS SUBJECTED DAILY TO SOLAR BOMBARDMENT. IN THE retina, pigments contained in photoreceptor cells further concentrate light causing extensive free radical formation. Consequently, as the visual process proceeds, photoreceptors, specifically the more sensitive rods are damaged (1). Damage to the rod outer segments results in its rapid renewal which also produces more reactive oxygen species. Renewal is effected via a mechanism whereby discs are added at the base of the rod outer segments and degraded from the apex of the cell. Degradation is mediated by the retinal pigment epithelium which phagocytose these structures into unknown degradation products. Both solar bombardment and phagocytosis therefore results in the production of large quantities of free radicals and reactive oxygen species. Phagocytosis results in the production of free radicals presumably via a Fenton mechanism (2) involving transition metals released by the degradation process. The increase in free radical concentrations subject the retina to a condition known as oxidative stress. In other epithelial cells such as those in kidneys, one of the consequences of this condition is the production of a low molecular weight inducible protein called metallothionein (3) whose functions are largely unknown. Metallothionein (MT) synthesis have also been observed in cells exposed to heavy metals, other stress conditions, hormonal changes and interferon. Increased synthesis is due to increase in transcription.

Induction by heavy metals such as Zn, Cu and Cd appears to be mediated by a metal responsive element (MRE) in the upstream regulatory regions of MT genes (4). Oxidative stress brings about synthesis of metallothionein by a mechanism believed to be similar to induction of synthesis by heavy metals and appears to be mediated by still unindentified cellular factors. Conditions that induce metallothionein synthesis, such as high zinc concentration and oxidative stress are both present in the eye.

Using histochemical techniques, the protein was detected in the retinal pigment epithelium (RPE) of mice (5). Similarly, using Cd binding studies (6), metallothionein was found to be synthesized by normal human RPE cell culture exposed to the heavy metals Cd and Zn.

This study was undertaken therefore to 1) verify the existence of metallothionein in the eye by PCR techniques and 2) to determine if metallothionein synthesized by

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monkey retinas show the same degree of conservation as metallothionein from other sources.

Experimental

Materials

Maintenance and care of monkeys was in accordance with the NIH Guide for the use and care of laboratory animals. These experiments were conducted according to the ARVO resolution on the use of Animals in Research. The monkeys were bred at the National Institute of Health Tsukuba Primate Center for Medical Science, Tsukuba, Ibaraki prefecture, Japan.

The AMV Reverse Transcriptase kit was obtained from GIBCO BRL, Research Products Life Technologies, Inc. Reagents and kit for PCR and DNA sequencing were obtained from Bio Rad and IBI. Iodoacetic acid was purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Rainbow markers were obtained from Amersham. All other reagents for electrophoresis and other chemicals were purchased from commercial sources and were used without further purification.

Methods

1. Preparation of retinal homogenates

Eyeballs were obtained by enucleation and whole retinas were taken and stored at -70°C until use. All procedures for the preparation of retinal homogenates were done at 0-4°C. Each retinal sample was minced in 3 mL 0.05 M phosphate buffer-1% Triton X-100, pH 7.0, and homogenized by 10 strokes of a Potter Elvehjem glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged for 20 min at 4,000 X g in a Tomy RL-100 refrigerated centrifuge. The supernate was collected and divided into aliquots, then stored at -70°C.

Total protein concentration was determined by the Bicinchoninic acid method (7).

2. Carboxymethylation and SDS-PAGE

The crude homogenate was heat treated at 80°C for 5 min then centrifuged at 10,000 rpm for 5 min. The supernate was treated with 1 mM dithiothreitol and EDTA, then incubated in 1 M iodoacetic acid for 15 min. After neutralizing with NaOH, the carboxymethylated samples were run in 15% acrylamide gels for 4 hours at 20 mA. The samples were stained with 0.2% Coomasie Blue, then restained using the Sigma silver stain kit.

3. Isolation of total RNA from retina

Total RNA was isolated from retinal samples using the method of Chirgwin (8).

4. cDNA synthesis and PCR for detection of metallothionein genes in the retina

cDNA was synthesized using the AMV transcription kit with modifications in the volume described in the manufacturer's procedure. Briefly, a mixture containing deoxynucleotide triphosphates (dNTPs), reverse transcriptase (RT) buffer, KCl buffer, oligo dT, actinomycin D, storage buffer and 2 U reverse transcriptase with a total volume of 26.5 uL was prepared. The mixture was incubated at 37°C for 60 min, then the reaction was stopped by heating at 95°C for 5 min and cooled immediately. Optical density was measured to determine approximate amount of product synthesized. An aliquot of the RT product was amplified using the polymerase chain reaction (PCR) method. For the specific amplication of monkey metallothionein Class II (MT2) cDNA, the primers (9) used were:

sense-5'AAGTCCCAGCGAGCCCGTGT-3'

and *antisense*-5' CCAGGTTTGTAGAGGTCGCA-3'

Reactions were carried out in a Perkin Elmer Cetus Thermal Cycler. The following conditions were used: 95°C denaturation for 30 sec, 54°C annealing, 1 min and 72.°C extension, 30 sec, for 30-35 cycles. After PCR, an aliquot of the mixture was run in 2% agarose gels containing ethidium bromide. The metallothionein cDNA was detected by uv illumination of the ethidium bromide stained band.

5. cDNA sequencing of metallothionein II

For sequencing, the PCR product was run in low melting agarose gels. After electrophoresis, the product was cut then purified in a Nu-Clean D50 (IBI) column. Sequencing was done using a flourescense-based DNA autosequencer (Applied Biosystems Inc. Model 373A).

Results and Discussion

1. SDS-PAGE of carboxymethylated retinal proteins

The electrophoretic profile of the carboxymethylated retinal proteins is shown in Fig. 1 where the putative metallothionein band is indicated by the arrow. Approximate molecular weight by SDS-PAGE is <10 kd. However, even with silver staining the band shown is faint suggesting a low concentration of the protein in the retina.

2. Detection of metallothionein

Fig. 2 shows the band for the PCR amplified metallothionein cDNA as detected by UV fluorescence. The approximate molecular size of this gene is 300 base pairs. This molecular size is consistent with metallothionein class II from rhesus monkey kidneys.

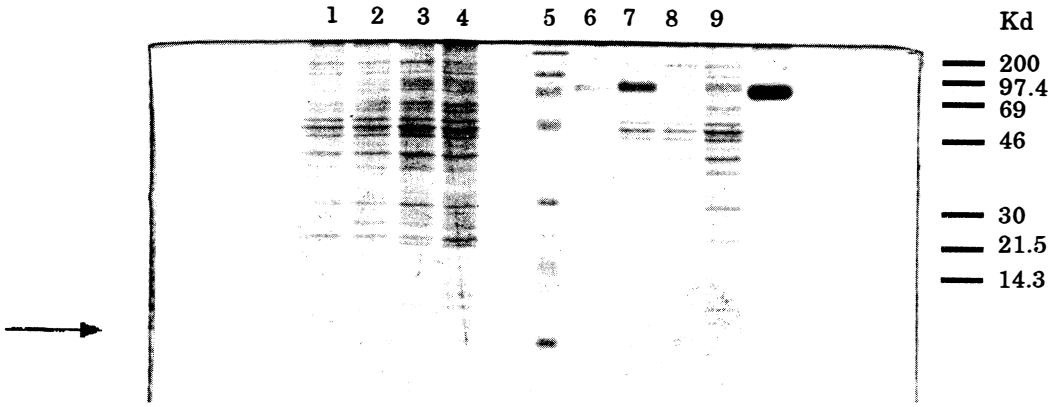

Figure 1. SDS-PAGE Profile of the carboxymethylated proteins of retina. Lanes 1-4, 7-9 are retinal homogenates from different families (lanes 3, 4, 9 are normal controls and lanes 3, 4, 7 and 8) cynomolgus monkeys. Lane 5 is the marker proteins.

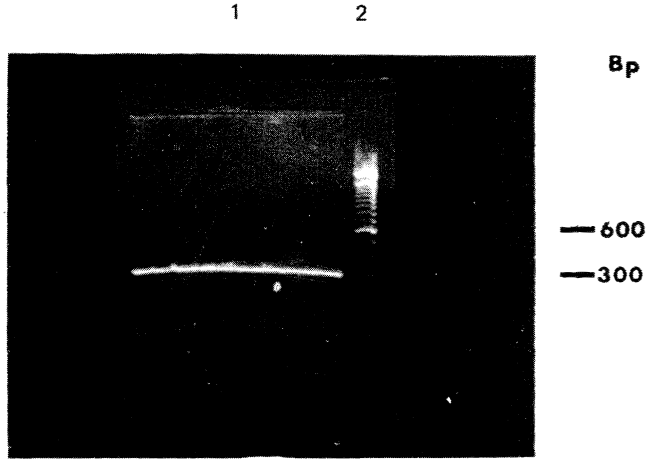

Figure 2. Metallothionein cDNA amplified by PCR. Lane 2 is the molecular weight marker (100 bp DNA ladder). Lane 1 is the metallothionein cDNA band which was subsequently purified for sequencing.

3. cDNA and amino acid sequence of the metallothionein gene from monkey retina

The cDNA and deduced amino acid sequence of the metallothionein gene from monkey retina is shown in Fig. 3. This protein detected from monkey retinas contains 20 Cys, 8 Lys, 7 Ser, no aromatic amino acids and belongs to the metallothionein class II. The metallothionein of cynomolgus retina has exactly the same amino acid sequence as that reported for monkey kidney cells (9).

The amino acid sequence of metallothionein from the *Macaca fascicularis* retina (see Fig. 4) also showed sequence similarity with metallothionein class II isolated from other sources (10).

Consequent to the daily renewal of rod outer segments is the accumulation of free radicals and a condition known as oxidative stress. Such condition is one of the factors known to induce metallothionein synthesis. Oliver and co-workers (6) detected metallothionein in retinal pigment epithelium. They have also shown its inducibility in these tissues.

Metallothionein is known to bind heavy metals such as zinc, copper and cadmium which also induce its synthesis. In the eye where zinc occurs at a very high concentration (11), the role of metallothionein is very important in maintaining this trace metal at a non toxic level and in supplying zinc-binding proteins and enzymes with the needed cofactor and/or prosthetic group. In addition, re-

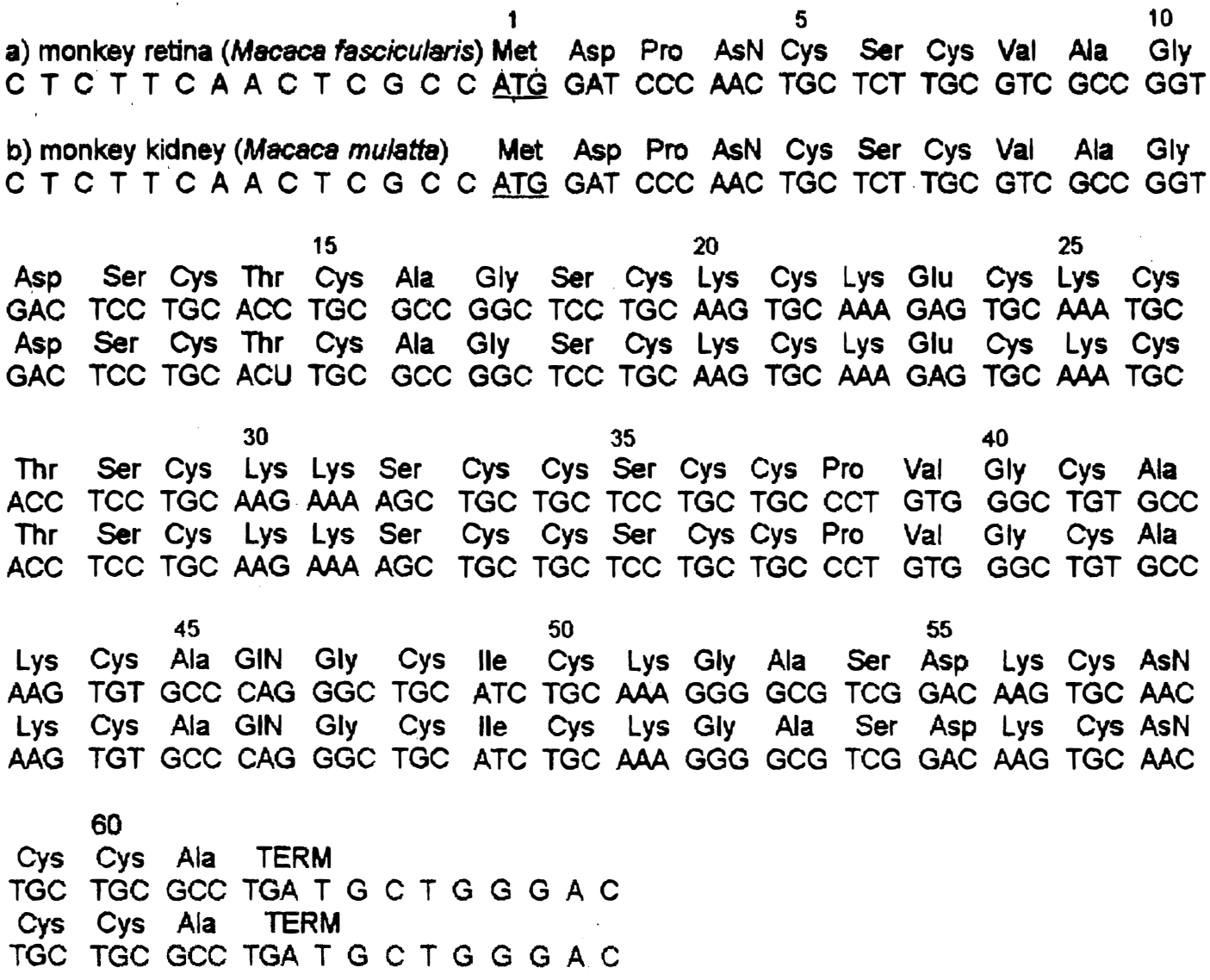

Figure 3. cDNA and deduced amino acid sequence for metallothionein from cynomolgus monkey retinas and similarity with metallothionein II from monkey kidney cells.
*The sequence corresponding to the initiation codon is underlined.

Sources	1				5					10					15		
monkey retina MT	M	D	P	N	C	S	C	V	A	G	D	S	C	T	C	A	G
monkey kidney MT2	M	D	P	N	C	S	C	V	A	G	D	S	C	T	C	A	G
human MT2	M	D	P	N	C	S	C	A	A	G	D	S	C	T	C	A	G
pigeon MT2	M	D	P	D	C	T	C	A	A	G	D	S	C	S	C	A	G

Sources			20					25					30				
monkey retina MT	S	C	K	C	K	E	C	K	C	T	S	C	K	K	S	C	C
monkey kidney MT2	S	C	K	C	K	E	C	K	C	T	S	C	K	K	S	C	C
human MT2	S	C	K	C	K	E	C	K	C	T	S	C	K	K	S	C	C
pigeon MT2	S	C	K	C	K	N	C	R	C	Q	S	C	R	K	S	C	C

Sources	35					40					45					50	
monkey retina MT	S	C	C	P	V	G	C	A	K	C	A	Q	G	C	I	C	K
monkey kidney MT2	S	C	C	P	V	G	C	A	K	C	A	Q	G	C	I	C	K
human MT2	S	C	C	P	V	G	C	A	K	C	A	Q	G	C	I	C	K
pigeon MT2	S	C	C	P	A	S	C	S	N	C	A	K	G	C	V	K	E

Sources				55					60								
monkey retina MT	G	A	S	D	K	C	N	C	C	A							
monkey kidney MT2	G	A	S	D	K	C	N	C	C	A							
human MT2	G	A	S	D	K	C	S	C	C	A							
pigeon MT2	P	S	S	S	K	C	S	C	C	H							

Figure 4. Sequence similarity of the amino acid sequence of metallothionein from cynomolgus monkey (*Macaca fascicularis*) retina with metallothionein Class II from other sources.

Abbreviations

MT: Metallothionein
RT: reverse transcriptase
PCR: polymerase chain reaction
dNTP: deoxynucleotide triphosphate
RPE: retinal pigment epithelium

cent studies have also shown that zinc binding to zinc-sensitive transcription factors control gene expression of metallothionein genes through the metal responsive elements (MRE) (4, 12) in the MT genes. Moreover, some investigators also believe that in tissues subjected to oxidative stress such as epithelial cells, metallothionein probably serves as a defense mechanism, scavenging free radicals to spare glutathione, another free radical scavenger.

Electrophoresis of carboxymethylated retinal proteins was able to detect a very faint band at a position corresponding to a molecular weight less than 10 kd, supposedly that of metallothionein. Carboxymethylation was necessary because metallothionein tends to aggregate due to the numerous -SH groups of cysteine, resulting in a diffused band in SDS-PAGE under reducing conditions. This behavior was observed in the study giving credence to the presence of metallothionein despite the very faint band. To verify the existence of metallothionein, total RNA was isolated and metallothionein cDNA was specifically amplified using as primers 20 mer sequences at the upstream and downstream region of the metallothionein cDNA class II from monkey kidney cells. Our results show the presence of a 300 base pair cDNA consistent with MT2 from monkey kidneys. In addition, cDNA sequence analysis of this protein showed a very high degree of similarity with the kidney MT2. Amino acid sequence analysis showed further similarity with MT2 from other sources.

These results show that metallothionein is indeed synthesized in retinas, perhaps as a response to oxidative stress as well as the presence of high concentrations of the heavy metal zinc. The cDNA and amino acid sequence obtained is also the first reported for metallothionein class II synthesized in the retina.

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