

## Induction of Cataract in Methylnitrosourea Treated Fischer (F344) Rats

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### ABSTRACT

Cataracts were observed in female F344 rats who intravenously received methylnitrosourea (MNU), a potent carcinogen for multiple organs, in one dose of 50 mg/kg body weight. Induction of cataract 40 weeks after MNU treatment was 41% whereas no cataract was observed in control rats. The aggregation of  $\beta_H$ -crystallin fraction by MNU was studied *in vitro*. HPLC pattern of  $\beta_H$ -crystallin changed when lens protein was incubated for 24 hrs with MNU. HPLC patterns indicated that MNU induced high molecular weight aggregates of  $\beta_H$ -crystallin. This study conveys some indication about the direct interaction of MNU with lens protein in cataract formation.

**Key words:** Rat, Methylnitrosourea, Cataract, Aggregation of  $\beta_H$ -crystallin

Cataracts are usually considered to be an aging phenomenon, but increasingly, ultraviolet, X-irradiation and various chemicals are thought to play a significant role in cataract formation<sup>5</sup>. Chemical modification of lens proteins is one of the important causes for cataractogenesis evidenced by different chemicals such as galactose, ethanol, cyanate, naphthalene, steroids<sup>7</sup>. Formation of certain types of cataract appears to be associated with the generation of HMW aggregates which may act as scattering points of light<sup>9,16</sup>. Comparative study of quantity and polypeptide compositions of the water-soluble and -insoluble proteins in normal and cataractous lens of the same age revealed that HMW aggregates increased at the expense of water-soluble protein<sup>6</sup>. In the bovine lens, water-insoluble protein is composed primarily of  $\alpha$ -crystallin<sup>10</sup>. However, in the human lens, this fraction may contain components from possibly all of the major structural lens proteins<sup>13</sup>. In galactose induced cataractous rat lenses, F-II- and  $\gamma$ -crystallin fractions became insoluble gradually<sup>11</sup>. However, the mechanism for the development of cataracts has not yet been fully elucidated. In this report, we have shown induction of cataracts in rats and also *in vitro* aggregation of  $\beta_H$ -crystallin fraction by MNU.

### MATERIALS AND METHODS

#### 1. Treatment of animals:

Two groups of rats (Charles River Ltd., Japan) were fed a commercial diet and water ad libitum.

Gr-1 (36 rats) was taken as a control group and observed for spontaneous cataract formation. Gr-2 (85 rats) at 7 weeks of age received MNU (Sigma Chemical Co., St. Louis, Mo.), dissolved in physiological saline, intravenously at a dose of 50 mg/kg body weight via jugular vein under light ether anaesthesia. Rats were observed maximally for 40 weeks.

#### 2. Histology of cataractous lens:

Whole eye containing cataractous lens was collected and fixed in a solution containing 10% formalin in PBS, ethanol, acetic acid and water at a ratio of 2:3:1:3 for 2 weeks. Fixed lenses were then embedded, sectioned and stained with haematoxylin-eosin for histological study.

#### 3. Isolation of crystallins:

Eye balls were enucleated immediately after sacrifice and kept in ice. Lenses were dissected out, decapsulated and kept at  $-80^{\circ}\text{C}$ . Crystallins were isolated chromatographically according to Siezen and Berger (1978)<sup>14</sup> using Sephadex G-200 (Pharmacia Fine Chemicals) column. Normal and cataractous lens extracts were applied separately on to the column equilibrated with Tris-HCl buffer, pH 7.3, and fractions were collected. Each fraction was monitored at 280 nm and different crystallin fractions were pooled, dialyzed against distilled water at  $4^{\circ}\text{C}$ , lyophilized and stored at  $-20^{\circ}\text{C}$  for subsequent studies.

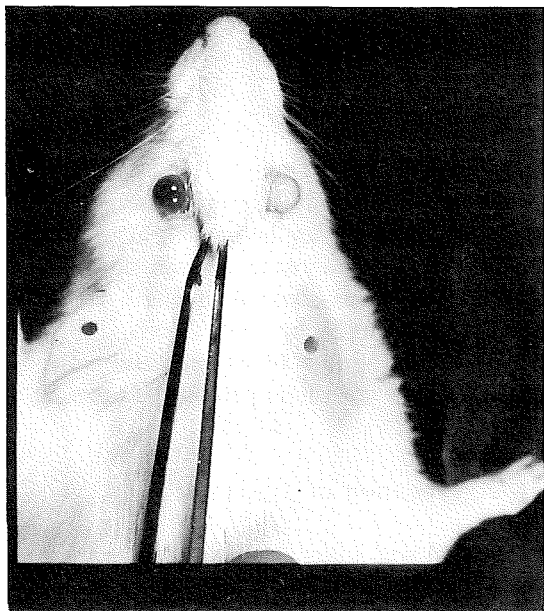
4. HPLC study for interaction of MNU and  $\beta_H$ -crystallin: Isolated  $\beta_H$ -crystallin was dissolved in PBS at a conc. 16.6 mg/ml and filtered through 0.2

**Table 1.** Occurrence of cataracts in methylnitrosourea treated F344 female rats

Group	Treatment	No. of rats examined	Cataracts (%)			Observation periods (mean) weeks.
			U	B	T	
1	—	36	0	0	0	36 (36)
2	MNU	85	9 (11)	26 (30)*	35 (41)*	17–40 (35)

U, B, and T: unilateral; bilateral and total.

\*: Gr-1 versus Gr-2 is significantly different by  $p < 0.01$ .

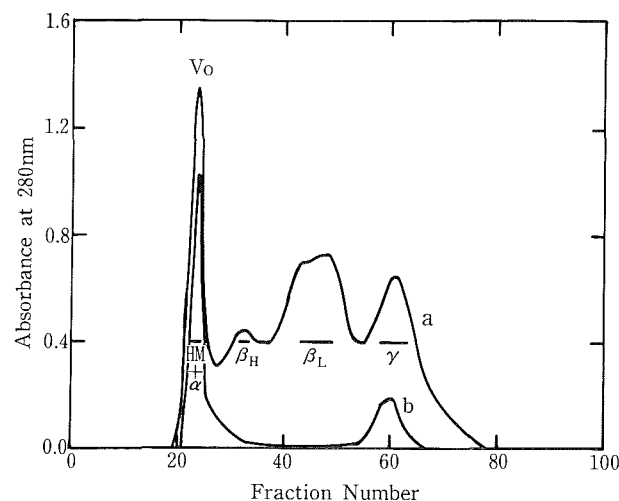


**Fig. 1.** Mature unilateral cataract found in a MNU treated rat after 33 weeks of MNU treatment.

$\mu\text{m}$  filter (Millipore). MNU was dissolved in PBS with shaking at a conc. 10 mg/ml and also filtered by 0.2  $\mu\text{m}$  filter. In order to obtain mixtures of crystallin and MNU at different weight ratio (viz. crystallin: MNU, 1:2.8, 1:1 and 1:0.28), stock crystallin and MNU solutions were proportionally mixed so that protein concentrations remained same (1 mg/ml) in all mixtures whereas MNU concentrations varied. Mixtures were incubated for different periods at 37°C and injected in HPLC column W550 (Hitachi Ltd., Japan), having a void volume range greater than  $2 \times 10^6$  and equilibrated with PBS, with the help of 25  $\mu\text{l}$  Hamilton syringe. Eluants were monitored with variable wavelength U.V. monitor at 280 nm and plotted by chromatointegrator (Hitachi Ltd., Japan) system attached to the column.

## RESULTS

Cataracts appeared in one or both eyes by an incidence of 41% in rats given MNU (11% unilateral and 30% bilateral) but no spontaneous cataract formation was observed in control rats within the observation period (Table 1). Occurrence of cataracts was noticed usually 33 weeks after MNU administration (Fig. 1). Our histological study of the cataractous lens showed that lens fibers became degenerated, fragmented and liquified. This has also been observed in other cataractous lens regardless

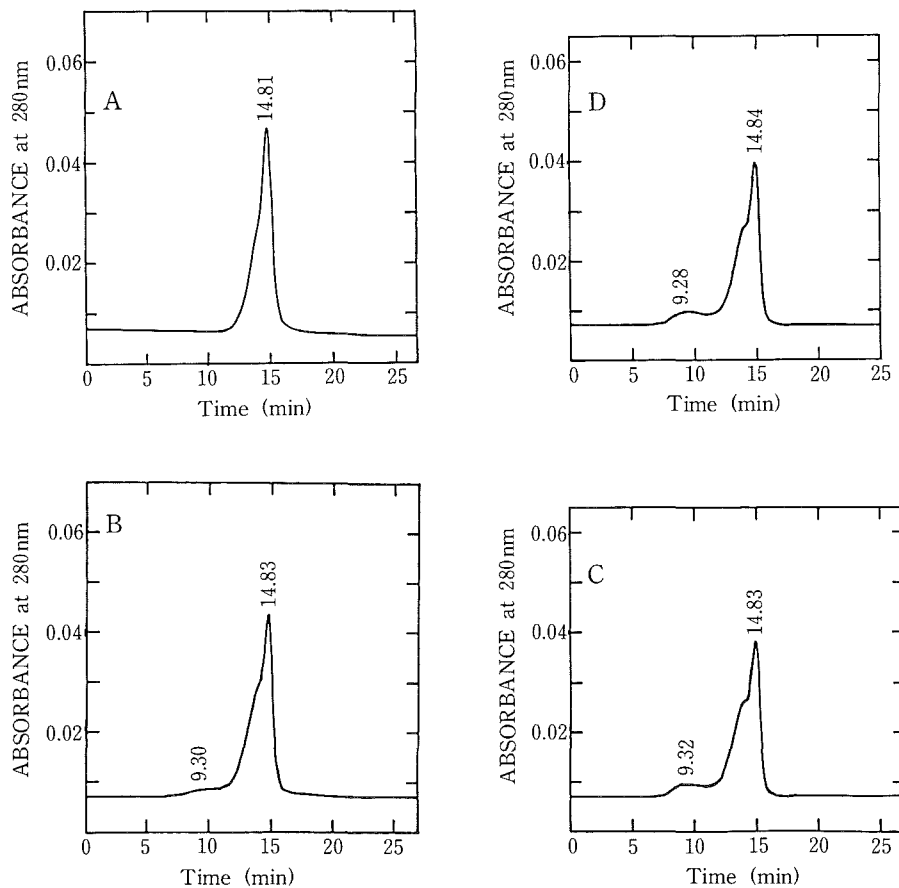


**Fig. 2.** Gel chromatographic patterns of lens extracts from normal (a) and MNU-induced cataractous (b) lenses on Sephadex G-200 column ( $2.5 \times 65 \text{ cm}^2$ ) equilibrated with Tris-HCl buffer, pH 7.3, at 4°C. Proteins of 83.5 and 29.3 mg (in 6.5 ml buffer) from normal and cataractous lenses were applied and 5.0 ml fractions were collected at a flow rate of 30 ml/hr.  $V_0$ , represents void volume of the column.

of causative agents<sup>4</sup>).

Fig. 2 showed elution patterns of normal and MNU induced cataractous rat lens extract by Sephadex G-200 at pH 7.3 and 4°C. The elution profile of normal lens extracts exhibited four separated peaks. They have been designated as a mixture of HMW aggregates of crystallins and  $\alpha$ -crystallin,  $\beta_H$ ,  $\beta_L$  and  $\gamma$ -crystallins, respectively, according to their elution behaviors<sup>12</sup>) and also characterized by molecular weights of their polypeptides by SDS-PAGE (unpublished data). The elution profile of cataractous lens extracts exhibited only two peaks, one in the void volume and the other in  $\gamma$ -crystallin region.

Fig. 3 showed HPLC patterns of  $\beta_H$ -crystallin and a mixture of  $\beta_H$ -crystallin and MNU incubated for 24 hrs at 37°C.  $\beta_H$ -crystallin is eluted at 14.81 mins with slight broadening at the base of the peak indicating presence of heterogeneity in the sample. A hump of aggregated  $\beta_H$ -crystallin appeared before the peak of  $\beta_H$ -crystallin (14.81 mins region) and became clearer as MNU concentrations increased from 0.28 to 2.8 mg/ml. Another HMW aggregate was also generated and eluted at 9.30 mins region.



**Fig. 3.** HPLC patterns of 0.0 (A), 0.28 (B), 1.0 (C) and 2.8 (D) mg/ml MNU treated (24 hrs)  $\beta_{\text{H}}$ -crystallin, respectively. 20  $\mu\text{l}$  samples were applied and elutions were carried at 1.0 ml/min and  $0.2 \times 100 \text{ kg/cm}^2$  pressure.

### DISCUSSION

The results indicate that cataracts were induced in rats treated with MNU but not in control rats. It is not clear why some cataracts were unilateral although the same phenomena have been observed with other causative agents<sup>5</sup>). One possibility might be that we failed to mark bilateral phenomenon macroscopically. Elution profile of cataractous lens extracts (Fig. 2) exhibited an absence of  $\beta_{\text{H}}$ - and  $\beta_{\text{L}}$ -crystallins. It might be that these two crystallin species had been converted to HMW species and ultimately to insoluble protein fraction as was observed in other species of animals<sup>10,13</sup>).

HPLC study revealed that MNU could not induce HMW aggregates within 4–5 hrs (data not shown) but could do so after 24 hrs of incubation. Observation of elution patterns of mixtures of  $\beta_{\text{H}}$ -crystallin and MNU (Fig. 3) made clear that formation of HMW aggregates of  $\beta_{\text{H}}$ -crystallin was induced by MNU in a dose dependent manner since MNU has no absorption at 280 nm at these concentrations. These HMW aggregates became distinct when MNU concentration was increased to 2.8 mg/ml. There are reports that crystallins tend to aggregate at higher concentrations<sup>2,15</sup>). The protein concentration used in this study was 1.0 mg/ml which is far below the self-aggregational concentra-

tion of crystallin observed by other workers. MNU used in this study contained acetic acid as an MNU stabilizer. We also checked the change in the pH of PBS due to acetic acid present in MNU. The maximum change in pH was found to be 0.2 units below the pH of PBS. It was observed that this small change in pH did not affect elution patterns of  $\beta_{\text{H}}$ -crystallin. In this context our results could be interpreted as showing the effect of MNU. Although our *in vitro* experiment showed that  $\beta_{\text{H}}$ -crystallin could be aggregated by MNU at a concentration of 0.28 mg/ml we still do not know the concentration of MNU in eye lens needed for cataract induction.

Experimental cataracts could be demonstrated by different DNA alkylating agents. Busulfan, an alkylating anticancer drug, induced cataracts in 8.9% of eyes of treated rats<sup>3,8</sup>). N-butyl-N-(4-hydroxybutyl) nitrosoamine, a bladder carcinogen induced cataracts in 15.7% of treated rats<sup>5</sup>). Administration of carcinogen may cause nutritional deficiency<sup>8</sup>) which will lead to cataract induction. In this study we attempted to explore the possibility of direct action of MNU with lens proteins rather than the secondary effect of MNU. Our results showed that MNU interacted with  $\beta_{\text{H}}$ -crystallin to induce the formation of HMW ag-

gregate which might play an important role in MNU induced opacity in rat lens. Therefore, the absence of  $\beta$ -crystallin in cataractous lens extract (Fig. 2) could be explained by the observation that aggregated  $\beta_H$ -crystallin became insoluble gradually. Alkylation of DNA by MNU is a well known phenomenon<sup>1)</sup>, but to our knowledge, the interaction of protein, peptide or amino acid with MNU has been little studied. In this respect, this study may initiate investigation of the interaction of alkylating agent with lens proteins.

#### ACKNOWLEDGEMENT

This work was supported in part by Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

(Received January 9, 1989)

(Accepted May 26, 1989)

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