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"POLY(ADP-RIBOSE)-SENSITIVE ENDONUCLEASE" AND THE REPAIR OF DNA DAMAGED BY N-METHYL-N-NITROSOUREA

H. Nomura[1], Y. Tanigawa, A. Kitamura, K. Kawakami[2] and M. Shimoyama

Department of Biochemistry, Shimane Medical University, Izumo, Shimane 693, Japan

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SUMMARY

When rat or hen liver nuclei were incubated in the presence of N-methyl-N-nitrosourea (MNU), there was a marked stimulation of the [^{3}H]dTMP incorporation into acid-insoluble fraction. When the incubation was carried out in the presence of MNU plus NAD, the effects of MNU on the DNA synthesis were nil. Furthermore, the incubation of heat-treated nuclei with either partially purified Ca^{2+}, Mg^{2+} - dependent endonuclease from rat liver or Mg^{2+}-dependent endonuclease from hen liver (sensitive to poly(ADP-ribose) formation), resulted in MNU-induced stimulation of the DNA synthesis. We propose that the "poly(ADP-ribose)-sensitive endonuclease" may participate in the DNA metabolism in cells exposed to DNA damaging agents.

Recent investigations on the physiological role of poly(ADP-ribose) have dealt with the participation of DNA metabolism. Smulson and associates (1,2), Shall and co-workers (3-5) and Jacobson et al. (6-8) reported independently that incubation of various cells with MNU[3] and other DNA damaging agents resulted in an increase in poly(ADP-ribose) synthetase activity concomitant with a decrease in the intracellular NAD levels and that inhibition of poly(ADP-ribose) synthetase by such agents as 5-methylnicotinamide, nicotinamide, and theophylline protects against the decrease in NAD levels. Similar results were also obtained by γ-

1. Part of this work will be included in the dissertation for the degree of Doctor of Medical Science.
2. On leave from the Department of Urology, Shimane Medical University.
3. The abbreviations used are: MNU, N-methyl-N-nitrosourea; TCA, trichloroacetic acid.

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radiation treatment of the cells (4,5). Thus, several investigators proposed that poly(ADP-ribose) may play a role in DNA repair (1-8).

Concerning the effect of poly(ADP-ribose) formation on DNA metabolism, Koide and associates (9) showed that ADP-ribosylation of Ca^{2+}, Mg^{2+} -dependent endonuclease is accompanied by an inhibition of DNA fragmentation to produce the primer for DNA polymerase activity. If the endonuclease that is inhibited by poly(ADP-ribose) formation is involved in the DNA metabolism during MNU treatment, it is quite probable that inhibition of poly(ADP-ribose) synthesis may result in an extensive degradation of DNA and subsequently potentiate the cytotoxicity of the agent.

In our studies on the effect of poly(ADP-ribose) on DNA metabolism, we observed that the partially purified endonuclease from hen liver nuclei was also inhibited by the poly(ADP-ribose) molecule[4]. In contrast to the rat liver enzyme, the partially purified hen liver enzyme depends on Mg^{2+} alone[4]. Using the nuclei from both rat and hen livers, we have found that "poly(ADP-ribose)-sensitive endonuclease"[5] is probably involved in the repair of DNA damaged by MNU.

MATERIALS AND METHODS

White male Wistar rats (200 g) and Rhode Island Red hens (1500 g) were maintained on an Oriental Yeast solid diet and tap water ad libitum. [Thymidine methyl-^{3}H]dTTP was obtained from New England Nuclear. DNA polymerase prepared from M. Lysodeikticus was purchased from Miles Laboratories, and NAD was obtained from Sigma Chemical Company. All other reagents were purchased from Miyata Chemicals Co. Ltd, Shimane.

Preparation of nuclei

The preparation of the nuclei was as described elsewhere(10). The final nuclear pellet was suspended in medium containing 30% glycerol, 50 mM Tris-Cl^- buffer (pH 8.0), 0.5 mM EGTA, 1 mM

4. Y. Tanigawa and M. Shimoyama, in preparation.
5. The endonuclease which is inhibited by poly(ADP-ribose) molecule or poly(ADP-ribosyl)ation of the enzyme protein has tentatively been referred to as poly(ADP-ribose)-sensitive endonuclease.

EDTA, and 2 mM 2-mercaptoethanol. The DNA in the nuclei was determined as described by Burton (11).

Activation of template activity of nuclei in the presence of MNU and NAD

The reaction mixture for activation of template activity of the nuclei contained 50 mM Tris-Cl^- buffer (pH 8.0), 10 mM $MgCl_2$, 6.25 mM $CaCl_2$, and 1 mM dithiothreitol, with or without 5 mM NAD, and/or 50 mM MNU, and appropriate amounts of the nuclei, in a total volume of 0.2 ml. The mixture was incubated at 37° C for various times. After incubation, the DNA polymerase assay was carried out for determination of template capacity of the nuclei (12).

DNA polymerase assay

The standard reaction mixture for the assay of DNA synthesis contained 30 mM Tris-Cl^- buffer (pH 8.0), 10 mM $MgCl_2$, 25 mM KCl, 2 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM ATP, 20 μM each of dATP, dGTP, dCTP, and [^{3}H]dTTP (0.5 μCi/4 nmoles), appropriate amounts of the nuclei, and 1 unit of M. Lysodeikticus DNA polymerase in a total volume of 0.2 ml. The mixture was incubated at 25° C for 15 min and stopped by the addition of 2.5 ml 10% TCA. The acid insoluble radioactive material was collected on a glass filter. The radioactivities of the sample were determinated using a Packard liquid scintillation spectrometer.

RESULTS AND DISCUSSION

As shown in Fig. 1, when rat liver nuclei were incubated with 50 mM MNU in the presence of both Ca^{2+} and Mg^{2+}, there was a marked increase in the incorporation of [^{3}H]dTMP into the acid-insoluble fraction, catalyzed by exogenous DNA polymerase. When the experiments depicted in Fig. 1 were done using endogenous DNA polymerase, the results were similar but the rate of [^{3}H]dTMP incorporation was low (data not shown). Furthermore, when the nuclei were incubated with MNU in the absence of Ca^{2+}, Mg^{2+}, or both of these compounds, there was no evidence of stimulation of the DNA synthesis by MNU (data not shown). The amount of MNU used was high compared to the amounts used in cell culture experiments (1,5), nevertheless, this is the first observation in isolated nuclei where DNA synthesis is enhanced by MNU.

When the nuclei were incubated in the presence of both MNU and NAD, stimulation by MNU of the DNA synthesis was not detected (Fig. 1). These results were shown to be specific for the

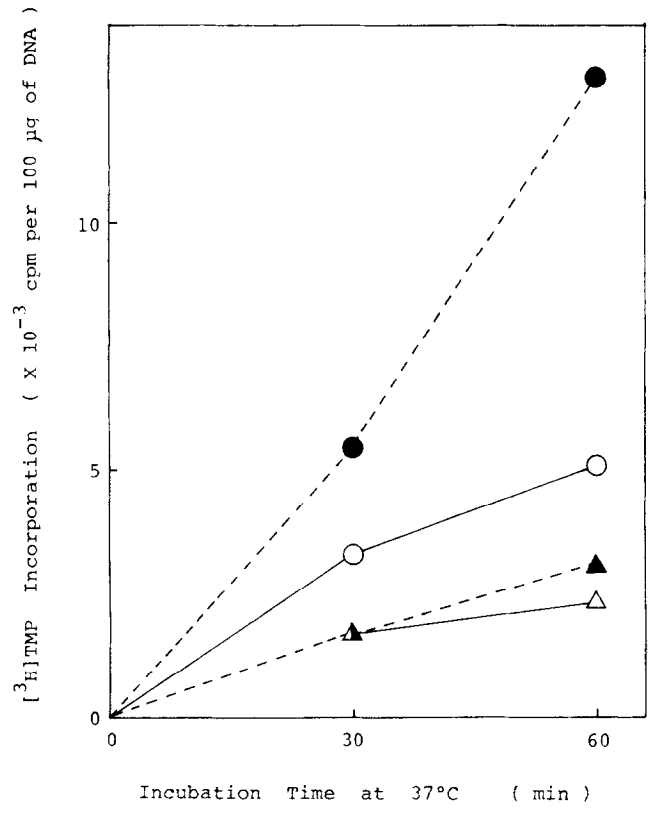

Fig. 1. Effect of MNU treatment of the rat liver nuclei, with or without NAD, on the DNA synthesis. The rat liver nuclei (171 µg DNA) were incubated with 50 mM MNU in the presence and in the absence of 5 mM NAD at 37° C for the indicated times. After incubation, the DNA polymerase assays were carried out as described in the Methods. o——o, none; ●----●, 50 mM MNU; △——△, 5 mM NAD; ▲---- ▲ , 50 mM MNU plus 5 mM NAD.

formation of poly(ADP-ribose) in the nuclei, as evidenced by inhibition with 20 mM nicotinamide (Fig. 2). The results shown in Figs. 1 and 2 together with the evidence of inhibition of Ca^{2+}, Mg^{2+} - dependent endonuclease by poly(ADP-ribose) formation reported by Koide and associates (9) indicate that the endonuclease probably participates in the damaged DNA repair processes. For clarification, the following experiments were carried out.

The nuclei were heated at 75° C for 15 min to inactivate the Ca^{2+}, Mg^{2+} - dependent endonuclease, and then were preincubated with or without MNU for 120 min at 37° C. After preincu-

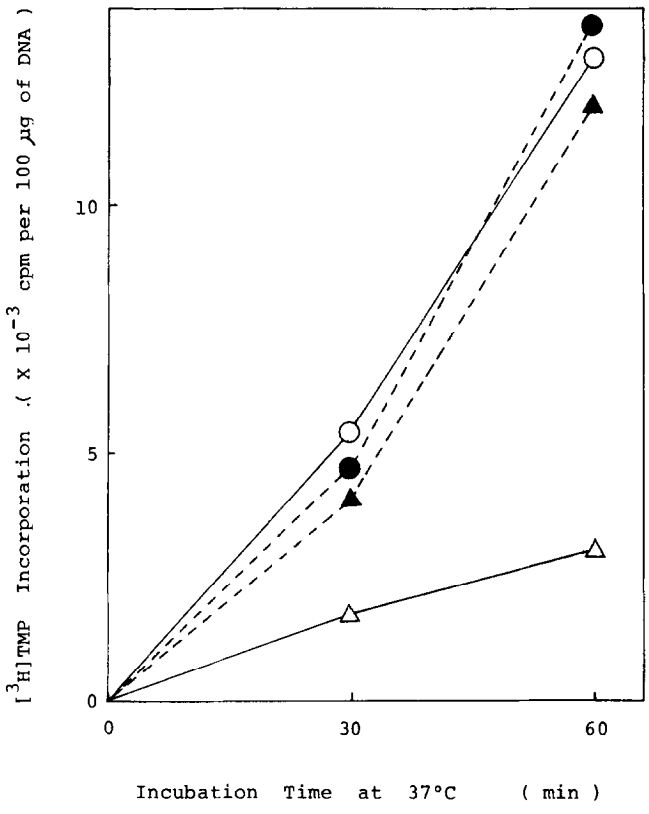

Fig. 2. Effect of nicotinamide treatment of the rat liver nuclei, with or without NAD in the presence of MNU, on the DNA synthesis. The incubation was carried out as described in the legend to Fig. 1 except that 20 mM nicotinamide were added to the incubation mixture. o——o, 50 mM MNU alone; △—— △ , 50 mM MNU and 5 mM NAD; ●----●, 50 mM MNU and 20 mM nicotinamide; ▲----▲, 50 mM MNU, 5 mM NAD and 20 mM nicotinamide.

bation, the mixture was centrifuged to remove MNU not bound to the nuclei. These nuclei were then incubated in the presence and absence of the partially purified Ca^{2+}, Mg^{2+} - dependent endonuclease. When the incubation was carried out in the absence of endonuclease, there was no effect of MNU on the [^{3}H]dTMP incorporation (Fig. 3). In the presence of endonuclease, however, MNU stimulation of DNA synthesis was evident as shown in the Fig. 1 experiment (Fig. 3).

Recently, we observed that hen liver nuclei contain Mg^{2+}-dependent endonuclease which is inhibited by poly(ADP-ribose)

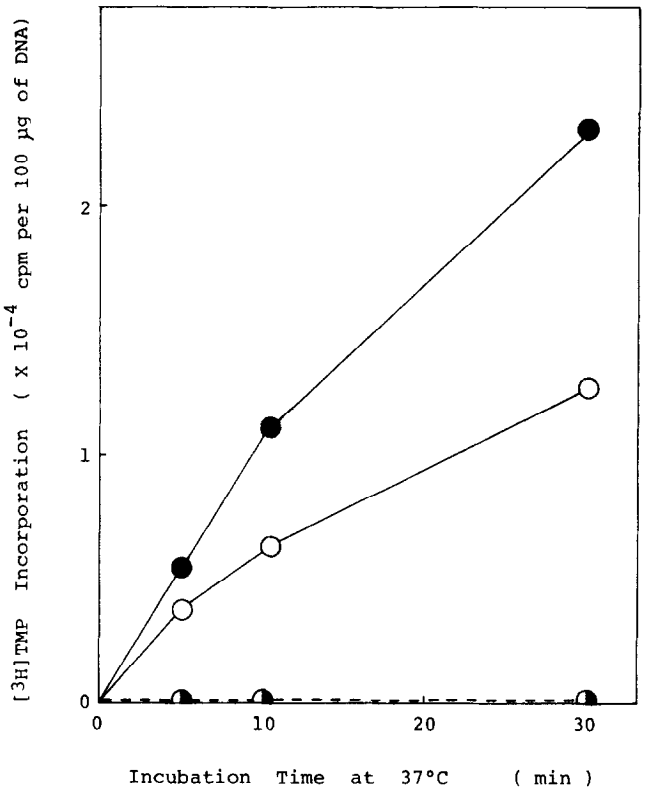

Fig. 3. MNU stimulation of DNA synthesis in the heat-treated nuclei with partially purified Ca^{2+}, Mg^{2+} - dependent endonuclease. The rat liver nuclei were heated at 75° C for 15 min. The nuclei were then preincubated with or without 50 mM MNU at 37° C for 120 min. The mixture was centrifuged at 10,000 x g for 10 min and these nuclei were then incubated at 37° C for the indicated times with appropriate amounts of the partially purified Ca^{2+}, Mg^{2+} - dependent endonuclease prepared from rat liver nuclei. Partial purification of the endonuclease from the rat liver nuclei were carried out through salt extraction, ammonium sulfate fractionation and DNA cellulose column chromatography, as described by Yoshihara et al. (9). Other conditions were as described in the legends to Figs. 1 and 2. ●, preincubated with MNU; o, preincubated without MNU; ———, incubated with endonuclease; – – – –, incubated without endonuclease.

molecules and that these nuclei contain no Ca^{2+}, Mg^{2+} - dependent-enzyme activity[4]. Therefore, it was of interest to determine whether MNU treatment enhances the [^{3}H]dTMP incorporation into the acid-insoluble fraction of the hen liver nuclei. As shown in Table I, the results obtained in the absence of Ca^{2+} were in good agreement with those from rat liver nuclei shown in Figs. 1 and

Table I. Effect of MNU treatment of hen liver nuclei with or without NAD, nicotinamide, or both on the DNA synthesis

Incubation in the presence of :	[^{3}H]dTMP incorporation	
	x 10^{-3} cpm/100 μg DNA	%
None	1.74	100
50 mM MNU	5.12	294.3
50 mM MNU + 5 mM NAD	0.81	46.6
50 mM MNU + 20 mM nicotinamide	3.78	217.2
50 mM MNU + 5 mM NAD + 20 mM nicotinamide	4.19	240.8

The hen liver nuclei (152 μg DNA) were incubated with MNU at 37° C for 120 min in the presence of indicated amounts of NAD, nicotinamide or both. The incubation mixture contained 10 mM Mg^{2+}, but not Ca^{2+}. Other incubation conditions were as described in the legends to Figs. 1 and 2. The nuclei incubated were then used for DNA polymerase assay as described under "Methods".

2. Furthermore, we confirmed the significant MNU effects on the DNA synthesis in the combined system of the partially purified Mg^{2+} - dependent endonuclease from the hen liver nuclei to the heat-treated nuclei, as shown with rat liver enzyme in Fig. 3 experiments (data not shown).

Thus, "poly(ADP-ribose)-sensitive endonuclease" probably induces a significant breakage in the DNA, preferentially in nuclei which have undergone MNU-modification. If this is indeed the case, treatment of cells with MNU brings about a fragmentation of DNA and subsequently stimulates poly(ADP-ribose) formation from NAD. Breakage of DNA may be reduced as a consequence of the inactivation of "poly(ADP-ribose)-sensitive endonuclease". In the presence of poly(ADP-ribose) synthetase inhibitor, however, the fragmentation of DNA by the endonuclease may continue at a rate higher than the rates of DNA repair. In fact, Smulson et al. (1) reported that inhibition of poly(ADP-ribose) synthetase with nicotinamide results in an extensive

degradation of the MNU-damaged DNA. In addition, Shall and co-workers (5) demonstrated the potentiation by 5-methylnicotinamide of the cytotoxicity of the DNA damaging agents. The evidence reported here is inconsistent with the findings of Durkacz et al. who suggested the relationship of poly(ADP-ribose) metabolism in processes of DNA excision repair in L1210 cells (13). Furthermore, our observations contribute to an understanding of the remarkable effects of nicotinamide on DNA metabolism in the presence of carcinogen (14, 15).

During the preparation of this manuscript, Edwards and Taylor (16) reported that poly(ADP-ribose) may function in the recovery from DNA damage by suppressing DNA synthesis. Using purified enzyme and cell systems, we are now attempting to provide direct and supportive evidence for our hypothesis.

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