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INCORPORATION OF NAD INTO FIXED CELL NUCLEI

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ABSTRACT

Incorporation of NAD into the nuclei was determined autoradiographically in cultured HeLa cells and in cryostat sections of rat organs by incubating them with ^3H -NAD after fixation with various agents. Acetone fixation was the best to render the cells permeable to NAD while preserving the cell's enzymatic activity to incorporate NAD into nuclear macromolecules. Various evidence supported that such incorporation of NAD is due mostly to the synthesis of poly(ADP-ribose) on chromatin proteins. In the sections of rat jejunum and esophagus the rate of NAD incorporation was higher in the actively proliferating cell nuclei than in the differentiated cell nuclei within the same epithelia. These results suggested that the capacity of the cells to synthesize poly(ADP-ribose) is associated with cell growth and differentiation.

INTRODUCTION

Poly(ADP-ribose) polymerase is a chromatin-bound enzyme which catalyzes synthesis of poly(ADP-ribose) from the ADP-ribose moiety of NAD (Hayaishi and Ueda, 1977; Purnell et al, 1980). Recently it has been suggested that the poly ADP-ribosylation of chromatin proteins is involved in the mechanism of DNA repair (Shall, 1984; Berger, 1985) as well as in certain categories of cell differentiation (Shall, 1983; Williams and Johnstone, 1983). To investigate the role of poly(ADP-ribose) polymerase in vivo, the in situ detection of the enzyme activity for individual cells seems useful. However, since living cell membrane is impermeable to externally added nucleotides, the poly(ADP-ribose) polymerase activity is usually measured by the uptake of radioactive NAD into macromolecules either in the isolated nuclei (Lehmann et al, 1974) or in the cells made permeable to NAD by cold hypotonic treatments (Berger et al, 1978; Skidmore et al, 1979). To my knowledge, autoradiographic attempts to detect incorporated NAD are found only in a few earlier reports. In such studies, ^3H -NAD was incorporated into isolated nuclei (Hilz and Kittler, 1968), unfixed tissue sections (Payne and Bal, 1976) or into the cells permeabilized by ammonium sulfate (Oikawa et al, 1969) and hence preservation of

morphological detail was insufficient. On the other hand, several laboratories reported using specific antibodies to poly(ADP-ribose) for the immunohistochemical detection of the enzyme activity (Ikai et al, 1980; Kanai et al, 1981; Ikai et al, 1982). It was shown herein that after the fixation of cells with acetone or alcohols, the cell nuclei remain capable of synthesizing poly(ADP-ribose) as demonstrated by the immunofluorescent staining.

In this report, I will show that acetone fixation of cultured cells or tissue sections readily renders the cells permeable to ^3H -NAD while preserving the cells' morphological integrity as well as capacity to synthesize poly(ADP-ribose), thus allowing autoradiographic detection of the poly(ADP-ribose) polymerase activity.

MATERIALS AND METHODS

Specimens. HeLa-S3 cells were cultured in an Eagle's minimum essential medium supplemented with 10 % calf serum on 18 x 18 mm coverslips placed in petri dishes. In some experiments, the cells were treated with various DNA-damaging agents added to the medium. The coverslips were rinsed with phosphate buffered saline prior to fixation. Male Wistar rats (6-8 weeks old, specified pathogen free) were sacrificed under ether anesthesia. The esophagus and the jejunum were removed and frozen immediately in OCT compound (Miles) dipped in dry ice/acetone. Cryostat sections were then made to 6 μm thickness and were air dried on slides for 20 min at room temperature prior to fixation.

Fixation. The following fixatives were used: acetone, 100 % ethanol, methanol/acetic acid (3:1), and 10 % formalin in 0.1 M phosphate buffered solution, pH 7.2. All fixations were carried out at 4 °C for 20 min. In certain experiments, the cells on coverslips were incubated with 0.5 % Nonidet P-40 (Shell) for 10 min after fixation.

Reaction condition. Following fixation, the cells on coverslips and the tissue sections were washed with 50 mM Tris-HCl, pH 8.0 and were incubated in 15 μl of the reaction mixture prepared according to Tanuma et al (1978), i.e., 0.37 μM of [adenine-2,8- ^3H] NAD (New England Nuclear, specific activity, 27 Ci/mmol), 15 mM MgCl_2 , 50 mM KCl and 10 mM 2-mercaptoethanol in 50 mM Tris-HCl, pH 8.0. In certain experiments, the cells were treated with DNase I (Cooper Biomedical) prior to the reaction. After incubation at room temperature, the reaction was terminated by rinsing the coverslips or slides with 50 mM Tris-HCl, pH 8.0. The coverslips were then treated with cold 5 % trichloroacetic acid (TCA) for 10 min and then dehydrated by ethanol. In certain experiments, the cells were treated after the reaction with various degradative enzymes, namely, DNase I, RNase A (Cooper Biomedical), pronase (Calbiochem) and snake venom phosphodiesterase (Boehringer Mannheim). In the case of tissue sections, the treatment with TCA was omitted from the procedure.

Autoradiography. The coverslips were mounted on slides with the cell surface up. The slides were dipped in an autoradiographic

emulsion (NR-M2, Sakura) and were exposed for 3 to 7 days at 4 °C. Following the development, the cells and tissue sections were stained with hematoxylin/eosine and the number of silver grains per nucleus was counted for 50 nuclei in each determination.

RESULTS

Incorporation of NAD into fixed cell nuclei. A significant number of silver grains, strictly localized to the nuclei, were obtained by autoradiography after the incubation of fixed HeLa cells with ^3H -NAD (Fig. 1). In acetone-fixed cells, the number of grains increased linearly within 20 min of the incubation period. With further incubation, however, the value was not reproducible between experiments (not shown). Ethanol fixation allowed the uptake of counts only one-seventh of that with acetone fixation. In contrast, after fixation in methanol/acetic acid or 10 % formalin, almost no count was obtained. This result was not caused by the difference in the permeability of cell membrane, because treatment of the cells with a detergent, Nonidet P-40, after any fixation procedure did not influence the subsequent uptake of NAD (not shown). In this regard, acetone fixation appears to be the best not only in making the cell membrane permeable to NAD but also in preserving the cells' enzymatic activity to incorporate NAD into nuclear macromolecules. Hence, in the subsequent experiments, the cells and tissue sections were routinely fixed in acetone and were subjected to the reaction immediately. A prolonged interval of time between the fixation and the start of reaction tended to cause elevation in the counts.

Characterization of incorporated counts. In the light of a number of earlier reports, it seems evident that the radioactivity incorporated from NAD into nuclear macromolecules is due to the poly ADP-ribosylation of chromatin proteins. In order to prove this in the present system, a set of experiments was carried out (Table 1). The incorporation of radioactivity abolished almost completely when the inhibitor of poly(ADP-ribose) polymerase such as

Fig. 1. Incorporation of NAD into HeLa cell nuclei after various fixations. Following treatment with the fixatives for 20 min, the cells were incubated with the ^3H -NAD mixture for the indicated periods. The incorporated radioactivity was determined after autoradiographic exposure of 3 days. ○, acetone; □, 100 % ethanol; Δ, methanol/acetic acid (3:1); ◇, 10 % formalin. Bars indicate mean $\pm$ SD of 50 nuclei counted.

Table 1. Effect of various treatments on incorporation of NAD into HeLa cell nuclei

Treatment			
Pre-incubation ^a	Reaction ^b	Post-incubation ^c	Grain counts ^d
NP	S	NP	59.2 ± 11.2
NP	3-Aminobenzamide	NP	0.4 ± 0.7
NP	Nicotinamide	NP	0.3 ± 0.4
NP	Nicotinic acid	NP	63.3 ± 17.0
DNase I, 10 ng/ml	S	NP	103.6 ± 24.3
100 ng/ml	S	NP	176.4 ± 30.0
1 µg/ml	S	NP	96.0 ± 20.8
NP	S	DNase I	41.9 ± 10.6
NP	S	RNase A	39.1 ± 11.3
NP	S	Pronase	2.8 ± 1.4
NP	S	Snake venom phosphodiesterase	0.7 ± 1.0

The acetone-fixed cells were incubated with the ³H-NAD mixture for 10 min under various conditions. ^a The cells were incubated with DNase I in 50 mM Tris-HCl, pH 8.0 plus 15 mM MgCl₂ for 30 min at 37 °C prior to the reaction. ^b The indicated agents were added at 5 mM to the standard reaction mixture presented in the Materials and Methods. ^c The cells were incubated with the digestive enzymes at 500 µg/ml in 50 mM Tris-HCl, pH 6.8 plus 20 mM MgCl₂ for 30 min at 37 °C after the reaction. ^d Mean ± SD of 50 nuclei² counted after 3 days of exposure. NP, not performed. S, standard condition.

3-aminobenzamide or nicotinamide (Sims et al, 1982) was present in the reaction mixture at a concentration of 5 mM. On the other hand, nicotinic acid, a derivative of nicotinamide lacking the inhibitory activity for the enzyme, did not affect the incorporation. Treatment of the fixed cells with DNase prior to incubation with NAD led to a marked increase in the uptake of radioactivity, in agreement with the fact that poly(ADP-ribose) polymerase is activated by DNase treatment of nuclei (Miller, 1975). Furthermore, post-incubation of the cells with pronase or snake venom phosphodiesterase rendered the counts entirely acid-soluble, while DNase or RNase caused a much smaller decrease in the counts. These results strongly support that the obtained counts are derived mostly from the formation of poly(ADP-ribose) on chromatin proteins during the incubation period.

Effect of DNA damaging agents. It is well established that poly(ADP-ribose) polymerase is activated by the formation of DNA strand breaks (Shall, 1984; Berger, 1985). A variety of agents that cause DNA damages are known to stimulate this enzyme activity, including dimethyl sulfate (Durkacz et al, 1980), Bleomycin (Miller, 1977), and neocarzinostatin (Iseki and Mori, 1985B). In the present study, HeLa cells in culture were treated with above agents for 30

Table 2. Effect of treatment with DNA damaging agents on incorporation of NAD into HeLa cell nuclei.

Agent, concentration	Grain counts ^a
Control	37.3 ± 9.3
Dimethyl sulfate, 60 µM	120.1 ± 29.0
Bleomycin, 250 µg/ml	181.3 ± 44.5
Neocarzinostatin, 25 µg/ml	114.6 ± 34.2

The cells were treated with the agents for 30 min prior to fixation. After incubation with ³H-NAD for 10 min, the incorporation of NAD was determined as in Fig. 1. ^a Mean ± SD of 50 nuclei counted.

min prior to acetone fixation and then the incorporation of NAD in 10 min was measured by autoradiography. As shown in Table 2, all these agents caused increases in the counts, suggesting that the obtained counts represent the level of poly(ADP-ribose) polymerase activity at the time of cell fixation.

Studies in rat tissue sections. There is evidence that change in the level of poly(ADP-ribose) polymerase activity is associated with the cell differentiation in several experimental systems (Farzaneh et al, 1982; Althaus et al, 1982; Johnstone and Williams, 1982). In the present study I intended to elucidate whether the present procedure was applicable to tissue sections to detect such association in vivo. The mucosa of the rat jejunum and esophagus were used for this purpose, because both the columnar epithelium of the former and the stratified squamous epithelium of the latter consist of actively dividing unmaturing cells on one hand, and the cells, derived from them, that have ceased to divide and have differentiated (Leblond, 1965). There is evidence that the structure of chromatin differs between the two cell populations in both tissues (Iseki and Mori, 1986). In the small intestinal mucosa of the guinea pig, the proliferating cells in the crypts of Lieberkuhn possess much higher activity of poly(ADP-ribose) polymerase than do the villus epithelial cells, as detected by the incorporation of NAD into isolated nuclei (Porteous et al, 1979). In the squamous epithelium of human skin, an immunohistochemical study using an antibody to poly(ADP-ribose) demonstrated that nuclei of the basal layer have higher level of the enzyme activity than do the keratinizing cell nuclei in the upper layers (Ikai et al, 1982). In the present study, cryostat sections of rat jejunum and esophagus were fixed in acetone and were examined for the incorporation of NAD in the same way as with HeLa cells, but without treatment with TCA to avoid morphological destruction. The time required from the sacrifice of the animal to the start of incubation was 60-90 min. As shown in Fig. 2, significant numbers of silver grains were observed in the nuclei of both epithelia. Such incorporation of radioactivity was specifically blocked by 3-aminobenzamide. The number of silver grains differed significantly between the two cell populations in both tissues (Table 3). In jejunal epithelium, crypt

cell nuclei incorporated approximately 5 times as many counts as did the whole villus epithelial nuclei on the average. The grains exhibited a gradual decrease in number as the cells went up through the villus and was almost absent near the villus tip. Also, in the esophageal epithelium the counts in the basal cell nuclei were 2.5 times that in the cells of upper layers on the average. These observations are consistent with the earlier reports and suggest the usefulness of the autoradiographic method in estimating the relative activity of cells to synthesize poly(ADP-ribose) in tissue sections. I also treated sections with DNase I (1 ng-1 mg/ml) prior to incubation with NAD but failed to obtain an increase in the counts (not shown). The reason for this result is unclear but will be discussed later.

Table 3. Incorporation of NAD into nuclei of rat tissues

Organ	Cell population	Number of nuclei counted ^a	Grain counts ^b
Jejunum	Crypt epithelium	120	6.3 ± 2.2
	Villus epithelium	155	1.3 ± 1.1
Esophagus	Basal layer	100	14.6 ± 6.5
	Upper layers ^c	166	6.0 ± 3.2

With the autoradiographs shown in Fig. 2, the number of grains per nucleus was determined for different cell populations. ^aAll the cells in a single villus and adjacent two crypts (for jejunum), and in a defined segment of epithelium (for esophagus) are counted. ^bMean ± SD. ^cThe spinous and the granular layers.

DISCUSSION

The autoradiographic detection of the incorporation of radioactive NAD has already been reported by several laboratories (Hilz and Kittler, 1968; Oikawa et al, 1969; Payne and Bal, 1976) but has not been established as a standard histochemical method to detect the poly(ADP-ribose) polymerase activity *in situ*. In the present study, acetone fixation of cells proved useful for this purpose. Ample evidence, obtained in the studies with HeLa cells, showed that the incorporated counts belong to poly(ADP-ribose) formed on chromatin proteins. The incorporation of NAD as detected by grain counts increased linearly for the initial 20 min or so but not after longer incubation periods. In regard of this, many factors are likely to be involved in the net amount of NAD incorporation: not only the synthesis of poly(ADP-ribose) but also degradation of it; initial enzyme activity as well as the activity induced by endogenous DNA damage during the incubation period. Furthermore, the existence of an artifact such as solubilization of acetone-precipitated material in the incubation medium can not be ruled out. Nevertheless, as far as being evaluated in a shorter incubation period, the initial rate of NAD incorporation as detected by grain counts seems to reflect the activity of poly(ADP-ribose) polymerase at the time of cell fixation.

The autoradiographic method proved also applicable to cryostat sections of rat tissues. In both the jejunal and esophageal epithelia, NAD was incorporated preferentially into the nuclei of

Fig. 2. Autoradiographic detection of incorporated ³H-NAD into cryostat sections of rat tissues. After fixation of the sections with acetone, the reaction was carried out for 10 min in the absence (A, C, E) and presence (B, D, F) of 3-aminobenzamide (5 mM). Exposure time was 7 days. The sections were stained with hematoxylin/eosine. (A, B), the crypt of jejunum, x360. (C, D), the villus tip of jejunum, x360. (E, F), esophageal mucosa, x720.

cells known to proliferate, suggesting the relationship of nuclear poly ADP-ribosylation with the cell growth and differentiation. The reason why DNase treatment of the tissue sections caused, contrary to the case of HeLa cells, no increase in the NAD incorporation requires consideration. According to Berger et al (1978), the incorporation of NAD can measure two types of poly(ADP-ribose) polymerase activity, one being the basal level of activity and the other the DNase-responsive or maximal activity. Hence, for the case of tissue sections, two interpretations are possible. One is that under the present conditions for preparation of tissue sections, i.e., freezing, cryostat sectioning and air drying, the cells have become incapable to activate poly(ADP-ribose) polymerase in response to DNA damages. The other is that the same conditions have caused the maximal activation of the enzyme so that the nuclei are no more responsive to DNase. If the latter interpretation is the case, the observed rate of NAD incorporation in tissue sections may represent the varying maximal capacity of the cells to activate the poly(ADP-ribose) polymerase, rather than the temporal enzyme activity in vivo. Further study will be required to clarify this point.

As a histochemical means of demonstrating poly(ADP-ribose), the immunofluorescence technique using the specific antibody has been reported (Ikai et al, 1980; Kanai et al, 1981). However, this technique detects the total amount of poly(ADP-ribose) rather than the rate of the synthesis. The net increase in the amount of the polymer can therefore be estimated only after comparing the fluorescence before and after incubation with NAD. Furthermore, the intensity of fluorescence is difficult to evaluate quantitatively. In this regard, the autoradiographic method will remain useful for semi-quantitative demonstration of the poly(ADP-ribose) synthesis in histological specimens, with morphological integrity maintained.

The poly(ADP-ribose) polymerase is activated by the formation of strand breaks in the DNA. Recently we reported the use of in situ nick translation procedure for demonstrating histochemically the single strand breaks of DNA in fixed cell nuclei (Iseki and Mori, 1985a). This technique is also applicable to cryostat sections of mammalian organs (Iseki, 1986). By combining the in situ nick translation procedure with present autoradiographic demonstration of the poly(ADP-ribose) synthesis, the means of investigating the role of DNA strand breaks in cell growth and differentiation will be expanded.

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