EFFECT OF DEOXYRIBONUCLEASE I ON THE NUMBER AND LENGTH OF CHAINS OF POLY(ADP-RIBOSE) SYNTHESIZED, *IN VITRO*

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Summary:
The effect of DNAase I on the synthesis of poly(ADP-ribose) by HeLa nuclei was studied. Experiments with the isolated products showed that the addition of DNAase I to the complete reaction mixture increased the chain length of the isolated product, poly(ADP-ribose), by only 10-20%. This small increase cannot account for the 400-600% increase in poly(ADP-ribose) polymerase activity that is seen when DNAase I is added to the assay media. So these results indicate that the main effect of DNAase I on the synthesis of poly(ADP-ribose) is through the initiation of new chains of poly(ADP-ribose). This evidence supports the idea that poly(ADP-ribose) polymerase may have a role in DNA repair.

Introduction:

Chambon and coworkers (1) reported in 1963 that a nuclear fraction from chicken liver catalyzed the incorporation of ATP into an acid-precipitable product. This reaction was markedly stimulated by NMN, and the product of the reaction was thought to be polyadenylic acid. Subsequent work by several groups (2-5) partially confirmed the basic findings of Chambon et al. (1); however, these investigators showed that the ATP had combined with NMN to formed NAD, and that the NAD was being utilized to form a new acid-precipitable polymer, poly(ADP-ribose)*. The enzyme responsible for the second reaction has been designated as poly(ADP-ribose) polymerase.

At this time the biological function of poly(ADP-ribose) polymerase is unknown; however, most of the data points to a close connection with nuclear DNA. The enzyme, as well as the product, is localized in the nucleus (3,6,7) and is firmly associated with nuclear proteins (8,9). These proteins, in turn, are bound to the nuclear DNA. Other results (10,11) have shown that the iso-

*Abbreviations used: ADP-ribose, adenosine 5'-diphosphoribose; DNAase I, pancreatic deoxyribonuclease; PR-AMP, 2'-(5"-phosphoribosyl)-5'-AMP.

lated enzyme is dependent upon DNA and histones for activity, while a positive correleation between the activity of poly(ADP-ribose) polymerase and the DNA content of various malignant and nonmalignant tissues has been established (12,13). Taken together, these results suggest that poly(ADP-ribose) polymerase may have a role in either DNA replication, DNA repair, or RNA synthesis.

Recent evidence both from this laboratory (14) and another (15) has also linked poly(ADP-ribose) polymerase activity to DNA. It has been shown that endonucleases can under the proper conditions stimulate the poly(ADP-ribose) polymerase activity of isolated nuclei. In this paper it will be shown that this stimulatory effect of endonucleases is through the initiation of new chains of poly(ADP-ribose). This finding strengthens the idea (14) that DNA fragmentation may be a necessary requirement for the synthesis of poly(ADP-ribose)

Materials and Methods:

Dowex 1-X2 (200-400 mesh), NAD, AMP, ADP-ribose, adenosine, pronase, and snake venom phosphodiesterase were obtained from Sigma Chemical Co., St. Louis, Mo. DNAase I (electrophoretically purified) was obtained from Worthington Biochemicals, Freehold, N.J., while [^{3}H]NAD was purchased from New England Nuclear, Boston, Mass. Hydroxylapatite, Bio-Gel HTP was obtained from Bio-Rad Laboratories, Richmond, Calif., while ATP was purchased from P-L Biochemicals, Milwaukee, Wisc. Scintisol was obtained from Isolabs Inc., Akron, Ohio.

HeLa cells were cultivated in 250-ml plastic bottles containing minimal essential media supplemented with Hanks basal salt solution, non-essential amino acids, 10% fetal calf serum, glutamine, and gentamicin. The cells, bottles, media, and supplements were purchased from Microbiological Associates, Bethesda, Md. The procedures for the isolation of HeLa nuclei, the measurement of poly(ADP-ribose) polymerase activity, and the isolation of poly(ADP-ribose) were described in an earlier publication (14). The remaining procedures will be described in the text.

Results and Discussion:

As indicated in an earlier publication (14) DNAase I can, under the proper conditions, stimulate the poly(ADP-ribose) polymerase activity of HeLa nuclei by 400-600%. This increase in activity was found (14) to be due to an increase in the synthesis of poly(ADP-ribose) and this result raises the question of how DNAase I increases the synthesis of this polymer. Is DNAase I stimulating the formation of new chains of poly(ADP-ribose) or the elongation of chains initiated *in situ*? To answer this question the products of the two assay systems (±DNAase I) were isolated (14) and compared. The first comparison utilized hydroxylapatite column chromatography. This technique is known to separate poly(ADP-ribose) according to chain length. There is

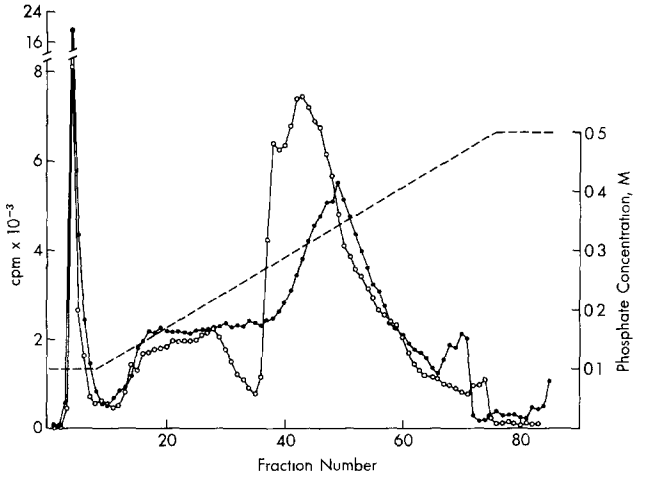

Fig. 1. Hydroxylapatite Column Chromatography of the Isolated Products. The reaction products were isolated as previously described (14). The products were fractionated in two separate runs on the same hydroxylapatite column (1 X 11 cm). The amount of radioactivity applied to the column for these separate determinations was the same (200,000 cpm). The recovery was 94% for the +DNAase I sample (O–O) and 97% for the -DNAase I sample (●–●). After the addition of either sample the column was rinsed with 20 ml of 0.1 M sodium phosphate buffer (pH 6.8), and eluted with a linear gradient formed using 75 ml of 0.1 M phosphate buffer and 75 ml of 0.5 M phosphate buffer (pH 6.8). Following the elution the column was rinsed with 20 ml of 0.5 M sodium phosphate buffer. The change in phosphate concentration is shown by the dashed line. The fraction size for the two runs was the same, 2.25 ml. The flow rate was also the same, 0.08 ml/min. This is the basic procedure of Sugimura et al. (16).

a linear relationship between the chain length of the poly(ADP-ribose) eluted and the phosphate concentration of the elution buffer (16). Figure 1 shows the results of this analysis. The radioactive profiles for the isolated products (±DNAase I) are very similar with two predominant peaks and a plateau between these peaks. There is some indication that the assay system with added DNAase I is synthesizing a slightly longer product (more radioactivity was found on the column in the second peak), and this was confirmed by using another technique.

Another way to look at the chain length of poly(ADP-ribose) is after a complete digestion with snake venom phosphodiesterase. This digestion will yield two predominant products, AMP and PR-AMP. These products can be

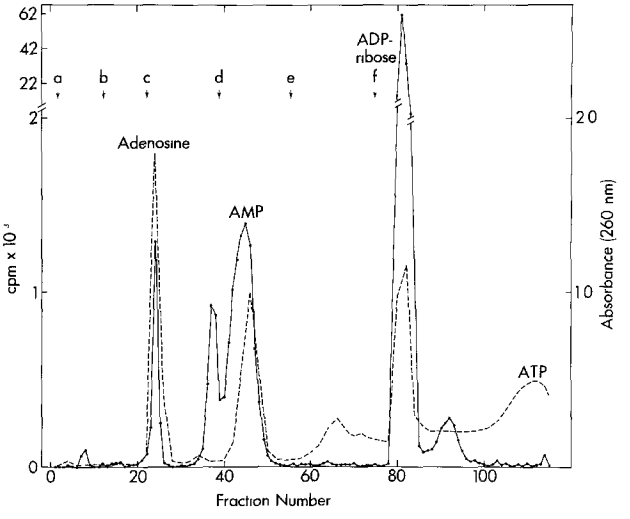

Fig. 2. Column Chromatography of the Hydrolysate of the +DNAase I Product by Snake Venom Phosphodiesterase. The incubation mixture contained 1.30 X 10^5 cpm of the isolated product (14), 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 1.2 mg of snake venom phosphodiesterase in a total of 4.0 ml. After 120 min. at 37°C, the mixture was applied with markers (adenosine, AMP, ADP-ribose, and ATP) to a Dowex 1-X2 formate column (1 X 25 cm). Elution was carried out in a step-wise manner with 30 ml of water (a), 30 ml of 0.05 M formic acid (b), 50 ml of 0.5 M formic acid (c), 50 ml of 1.0 M formic acid (d), 60 ml of 4.0 M formic acid (e), and 125 ml of 6.0 M formic acid and 0.4 M ammonium formate (f). Fractions of 3.0 ml were collected, and assayed for radioactivity (solid line) and absorbance at 260 nm (dashed line). The recovery of radioactivity was 100%. This is similar to the procedure of Shima et al. (17).

separated on a Dowex column. Figures 2 and 3 show the results of this type of analysis for the two products. Again the radioactive profiles for the two samples show a high degree of similarity. The major radioactivity peaks, AMP and PR-AMP, were eluted with the AMP and ADP-ribose markers. Three additional peaks of radioactivity were also detected. One eluted with the adenosine marker, the second preceded the AMP marker, and the third was found between the ADP-ribose and the ATP markers. The second minor peak may be 2'-(ribosyl)-5'-AMP or contaminating NAD from the assay (17), while the third peak is probably partially digested poly(ADP-ribose). The radioactivity under these three minor peaks accounts for less than 5% of the total radioactivity recovered from either column.

Besides indicating that the products from the two assay systems are similar, the data in Fig. 2 and 3 can be used to calculate the average chain length of the two samples of poly(ADP-ribose). This is done by comparing

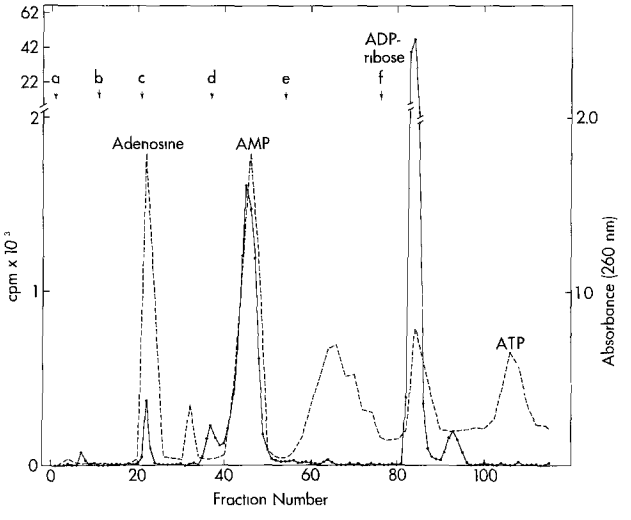

Fig. 3. Column Chromatography of the Hydrolysate of the -DNAase I Product by Snake Venom Phosphodiesterase. The isolated product (9.8 X 10^4 cpm) was treated with snake venom phosphodiesterase and applied to the same Dowex 1-X2 column. See Fig. 2 for experimental details. The radioactivity is represented by the solid line, while the absorbance at 260 nm is given by the dashed line. The recovery of radioactivity was 100%.

Table 1. Average Chain Length of the Isolated Products

System	Counts per minute AMP Peak (a)	PR-AMP Peak (b)	Average Chain Length (1 + b/a)
-DNAase I	7810	88700	12.4
+DNAase I	8710	116000	14.3

See Fig. 2 for experimental details.

the radioactivity under the two major peaks. The results of these calculations are given in Table 1. The average chain length was 12.4 for the -DNAase I sample and 14.3 for the +DNAase I sample.

Taken together the results of these two different analyses indicate the same thing. The products in both cases is poly(ADP-ribose), and the polymer from the +DNAase I assay is 10-20% longer than the polymer from the -DNAase I assay. This small increase in chain length cannot account for the 400-600% increase in poly(ADP-ribose) polymerase activity that is seen when DNAase I is added to the assay medium. So these two results indicate that the stimulatory effect of DNAase I on the poly(ADP-ribose) polymerase activity of HeLa nuclei is through the initiation of new chains of poly(ADP-ribose). This finding supports the idea that poly(ADP-ribose) polymerase may have a function in DNA repair since it indicates that DNA fragmentation is an initiation signal for the synthesis of poly(ADP-ribose).

These results also indicate that the synthesis of poly(ADP-ribose) occurs in a least three discrete steps. These steps are initiation, polymerization, and termination. Initiation is the attachment of the first unit of ADP-ribose to a nuclear protein. Polymerization is the elongation of the polymer, while termination is simply the cessation of polymerization. Since DNAase I only affects the first step in this three step process, it may be possible to use endonucleases under controlled conditions to define all the factors required for the initiation of poly(ADP-ribose).

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