

Poly(ADP-ribose) synthetase inhibitors enhance streptozotocin-induced killing of insulinoma cells by inhibiting the repair of DNA strand breaks

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1. INTRODUCTION

Streptozotocin, an antibiotic isolated from *Streptomyces achromogenes* [1], has diabetogenic [2], antitumoral [3] and oncogenic [4] activities. In our previous study on the diabetogenic action, we have shown that streptozotocin acts selectively on pancreatic islet cells to cause DNA strand breaks which stimulate poly(ADP-ribose) synthetase [5–8], a nuclear enzyme which has been suggested to participate in DNA repair [9,10].

The antitumoral action of streptozotocin is highly specific for insulinoma, and this agent has been successfully used for the treatment of human insulinomas [11,12]. In this paper, we present evidence that growth inhibition of hamster insulinoma cells due to streptozotocin is markedly enhanced by the poly(ADP-ribose) synthetase inhibitors such as nicotinamide and 3-aminobenzamide. Alkaline sucrose gradient analyses revealed that streptozotocin generates DNA strand breaks in the insulinoma cells, and that the inhibitor blocks the rejoining of DNA strand breaks.

2. MATERIALS AND METHODS

2.1. Materials

Streptozotocin was purchased from Upjohn Co., Kalamazoo, Michigan; nicotinamide from Wako

Pure Chemical Industries, Tokyo; [^3H]thymidine (6.7 Ci/mmol) from New England Nuclear, Boston; nicotinamide [U- ^{14}C]adenine dinucleotide ([^{14}C]NAD) (286 mCi/mmol) from Amersham International, Buckinghamshire; RPMI 1640 from Nissui Seiyaku, Tokyo; fetal bovine serum from Grand Island Biological Co., New York. 3-Aminobenzamide was kindly supplied by Dr. H. Nakano in Research Laboratories, Chugai Pharmaceutical Co., Tokyo.

2.2. Cells and culture conditions

Ins111R₁ cells are a cultured insulinoma cell line established from BK-virus-induced islet cell tumors of Syrian golden hamsters [13,14]. The cells were maintained in a monolayer culture at 37°C in RPMI 1640 medium supplemented with 15% fetal bovine serum and 50 µg/ml kanamycin [14].

2.3. Assay of islet nuclear poly(ADP-ribose) synthetase activity

About 2000 islets were isolated from rat pancreas by the method of Okamoto et al. [15]. Nuclear fraction was prepared from islets and assayed for poly(ADP-ribose) synthetase activity as described previously [6,8,16].

2.4. Measurement of cell growth

Ins111R₁ cells in log phase were seeded at a density of 5×10^4 cells/ml in culture tubes. After having been allowed to stand for 24 h to ensure attachment to the tubes, the cells were incubated in the presence or absence of streptozotocin with or

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Abbreviations: RPMI 1640, Roswell Park Memorial Institute Tissue Culture Medium 1640; EGTA ethylene glycol bis(β -aminoethyl ether)*N,N*-tetraacetic acid

without poly(ADP-ribose) synthetase inhibitors for various time intervals, and counted in the medium containing 0.02% (w/v) erythrocin B on a hemocytometer to estimate the number of viable cells [17].

2.5. Alkaline sucrose gradient analysis of DNA

In111R₁ cells were prelabelled for 20 h with 1 μ Ci/ml [³H]thymidine (1 μ M total thymidine). The radioactivity was washed out, and the cells were incubated in fresh medium in the presence or absence of streptozotocin with or without nicotinamide. After incubation, the cells were washed once with 0.9% cold NaCl, and 2×10^4 cells were then suspended in 50 μ l of 0.9% NaCl and immediately layered over 0.5 ml of lysis solution (1 N NaOH, 0.01 M EDTA, 1% (v/v) Triton X-100) that had just been layered over 14.8 ml of a 5–20% (w/v) linear sucrose density gradient containing 0.3 N NaOH, 0.7 M NaCl and 0.01 M EDTA [6–8]. On the bottom of the gradient was 1 ml of an 80% (w/v) sucrose shelf. The loaded gradients were placed in the dark at 20°C for 30 min to allow cell lysis, then centrifuged at 26 000 rev./min at 20°C for 3 h [6–8]. After centrifugation, fractions of 33 drops were collected from the gradient. DNA in each fraction was precipitated by adding 2 ml of 20% cold trichloroacetic acid with 200 μ g bovine serum albumin as carrier [6–8]. The precipitate was collected on a Whatman GF/C filter, washed 3 times with 5% cold trichloroacetic acid, once with ethanol, and then counted for ³H-radioactivity in a toluene-based scintillation solution.

3. RESULTS

As shown in fig.1, nicotinamide and 3-aminobenzamide inhibited poly(ADP-ribose) synthetase from rat pancreatic islets in a dose-dependent manner. The concentrations for 50% inhibition were estimated as follows; nicotinamide, 100 μ M; 3-aminobenzamide, 8 μ M. The enzyme activity almost completely inhibited by 10 mM nicotinamide or 1 mM 3-aminobenzamide. Poly(ADP-ribose) synthetase activity has been found ubiquitously among eukaryotic cells, and the enzyme has been suggested to be identical among tissues or cells from various mammalian species [18]. In fact, the enzyme from Chinese hamster ovary cells also was completely inhibited by 10 mM nicotinamide

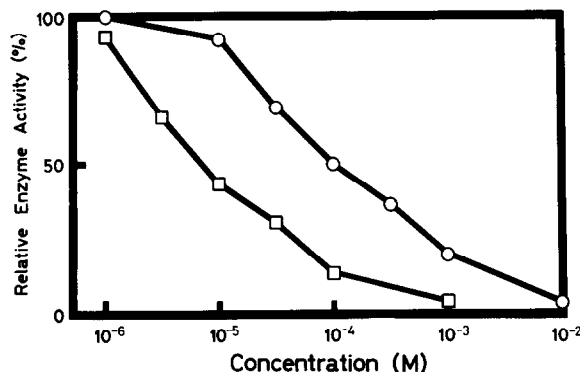


Fig. 1. Inhibition of islet nuclear poly(ADP-ribose) synthetase. Poly(ADP-ribose) synthetase activity was determined as described previously [6,8,16]. All activities were related to that of an equivalent quantity of islet nuclear fraction without inhibitor (57.3 pmol poly(ADP-ribose) synthesized $\cdot 10 \text{ min}^{-1} \cdot \mu\text{g}$ islet nuclear DNA⁻¹). Nicotinamide ($\circ$), 3-aminobenzamide ($\square$).

or 1 mM 3-aminobenzamide [19]. Therefore, we employed these inhibitors of the rat islet enzyme throughout the present experiments with hamster insulinoma cells.

We examined the effect of poly(ADP-ribose) synthetase inhibitors on streptozotocin-induced inhibition of hamster insulinoma cell (In111R₁) growth. As shown in fig.2, addition of 0.5 mg/ml streptozotocin inhibited In111R₁ cell growth, but the cells regained their exponential growth rate 3–5 days after the addition. However, together with 0.5 mg/ml streptozotocin and 10 mM nicotinamide, the viable cell number was found to be progressively decreased (fig.2a). 3-Aminobenzamide, the more potent inhibitor, enhanced the streptozotocin-induced inhibition of the insulinoma cell growth at the lower concentration (1 mM) (fig.2b). The poly(ADP-ribose) synthetase inhibitors per se had no effect on the growth of the cells at the concentrations used.

Streptozotocin has been shown in our previous study to cause DNA strand breaks in rat pancreatic islet cells [6,7]. Recently it was reported that inhibitors of poly(ADP-ribose) synthetase such as 3-aminobenzamide retard DNA repair from damages due to alkylating agents [10,20]. We therefore analyzed velocity sedimentation in an al-

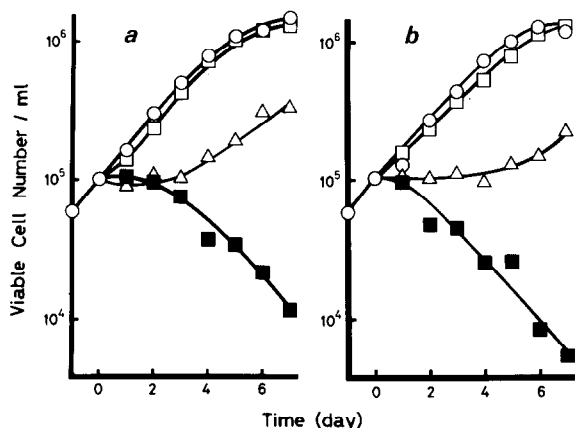


Fig.2. Effect of poly(ADP-ribose) synthetase inhibitors on streptozotocin-induced inhibition of In11R₁ cell growth. The chemicals were added on 0 day, before which the cells had been seeded at a density of 5×10^4 cells/ml, and had been cultured for one day to ensure their attachment to culture tubes. Each point represents the viable cell number/ml, which was determined as described in Materials and Methods. (a) In11R₁ cells incubated without any addition ($\circ$); with 0.5 mg/ml streptozotocin (Δ); with 0.5 mg/ml streptozotocin and 10 mM nicotinamide ($\blacksquare$); with 10 mM nicotinamide ($\square$). (b) In11R₁ cells incubated without any addition ($\circ$); with 0.5 mg/ml streptozotocin (Δ); with 0.5 mg/ml streptozotocin and 1 mM 3-aminobenzamide ($\blacksquare$); with 1 mM 3-aminobenzamide ($\square$).

kaline sucrose gradient of DNA from In11R₁ cells treated with streptozotocin alone or with streptozotocin and nicotinamide. In11R₁ cells were exposed to 0.5 mg/ml streptozotocin for 30 min. Immediately after the treatment, the DNA sedimented slowly as a broad peak in the middle of the gradient, regardless of the presence or absence of nicotinamide (fig.3a and b). As shown fig.3d, at 20 h after removal of streptozotocin the DNA was increased significantly in size to sediment fast near the bottom of the gradient, the position at which undamaged DNA sediments. However, when nicotinamide (10 mM) was present in the medium, the conversion of the low molecular weight DNA to a fast-sedimenting material was completely inhibited (fig.3c).

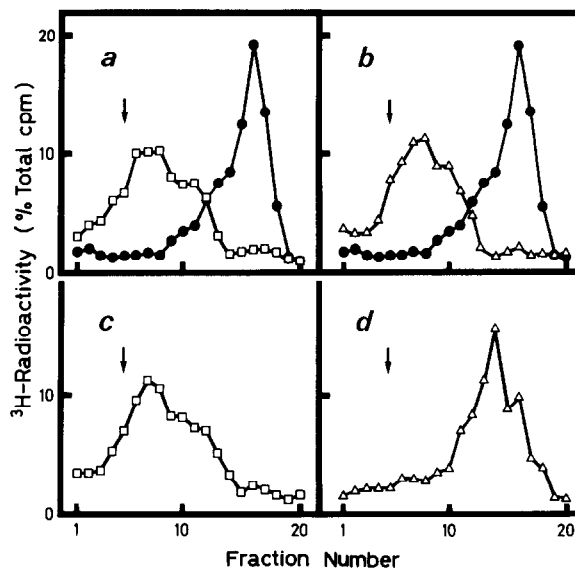


Fig.3. Effect of nicotinamide on rejoining of streptozotocin-induced DNA strand breaks in In11R₁ cells. The cells, which had been labelled with [3 H]thymidine, were treated with 0.5 mg/ml streptozotocin for 30 min in the presence or absence of 10 mM nicotinamide. Streptozotocin was then washed out, and the cells were further incubated for 20 h with or without 10 mM nicotinamide. After incubation, DNA of the cells was analyzed by velocity sedimentation in an alkaline sucrose density gradient as described in Materials and Methods. Each point represents % of total radioactivity recovered. The total radioactivity loaded onto the gradient was $1-2 \times 10^4$ cpm; recovery was between 85 and 95%. (a) DNA of In11R₁ cells incubated without any addition ($\bullet$); with 0.5 mg/ml streptozotocin and 10 mM nicotinamide ($\square$). (b) DNA of In11R₁ cells incubated without any addition ($\bullet$); with 0.5 mg/ml streptozotocin alone (Δ). (c) DNA of In11R₁ cells incubated with 10 mM nicotinamide for 20 h after removal of streptozotocin ($\square$). (d) DNA of In11R₁ cells incubated without nicotinamide for 20 h after removal of streptozotocin (Δ). Sedimentation was from left to right. Arrow indicates the position of a bacteriophage λ DNA (3.2×10^7 molecular weight, New England BioLabs).

4. DISCUSSION

The present study has clearly demonstrated that nicotinamide and 3-aminobenzamide, islet poly-(ADP-ribose) synthetase inhibitors, markedly enhance hamster insulinoma cell killing by strep-

tozotocin. This was supported by alternative evidence that the *in vivo* tumor-forming ability of the cells [14] is also completely abolished by a combined treatment with streptozotocin and nicotinamide (unpublished data, Yamamoto, H. and Okamoto, H.). Alkaline sucrose gradient study has revealed that streptozotocin generates DNA strand breaks in the insulinoma cells, and that nicotinamide inhibits the rejoining of the streptozotocin-induced strand breaks. Therefore, it is reasonable to assume that poly(ADP-ribose) synthetase inhibitors inhibit repair of the DNA strand breaks and hence enhance streptozotocin-induced inhibition of the insulinoma cell growth, while in the absence of the inhibitors the cells regain their ability to grow as the DNA recovers from the streptozotocin-induced breaks. In mouse leukemia cells poly(ADP-ribose) synthetase inhibitors were also observed to inhibit the rejoining step of DNA repair [10,20] although the inhibitors exhibited rather stimulatory effect on unscheduled DNA synthesis in normal cells such as rat hepatocytes [21] and human lymphocytes [22,23].

Shall and his colleagues have reported that cytotoxicity of dimethylsulfate or methylnitrosourea towards mouse leukemia cells was enhanced by poly(ADP-ribose) synthetase inhibitors [10,24]. Similar results were also observed in other rodent cell lines [25]. Caffeine may enhance X-ray-induced HeLa cell killing [26] or L-cell killing by alkylating agents [27] in a similar way, because caffeine also has been shown to inhibit poly(ADP-ribose) synthetase [28]. The enhancement by poly(ADP-ribose) synthetase inhibitors of cell killing effect of DNA damaging agents may have therapeutic potential in the treatment of human cancer.

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