

Opposite effects of cell differentiation and apoptosis on Ap₃A/Ap₄A ratio in human cell cultures

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Abstract The biological role of diadenosine oligophosphates (DAOP) remains obscure in spite of numerous attempts to solve this enigma. It is known that Ap₃A contrary to Ap₄A accumulates in human cultured cells treated with interferons (IFNs) alpha or gamma. Since IFNs are considered as antiproliferative regulators, we assumed that different cell status may be associated with varying intracellular levels of DAOP. Promyelocytic human cell line HL60 induced by phorbol ester (TPA) to differentiate to macrophage-like cells in culture exhibits a profound loss of proliferative potential. Here we have shown a 4–5-fold increase in Ap₃A concentration in HL60 cells induced by TPA, similar to the effect of IFN, while the Ap₄A concentration remained unchanged. On the contrary, in cells undergoing apoptosis induced by VP16, a topoisomerase II inhibitor, the Ap₃A concentration considerably decreased, while the Ap₄A concentration increased. These findings combined with earlier results suggest an involvement of the Ap₃A/Ap₄A ratio in signal transduction pathways controlling the cell status.

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1. Introduction

Aminoacyl-tRNA synthetases (ARS, E.C. 6.1.1) in the presence of ATP, ADP and substrate amino acids are synthesising diadenosine oligophosphates (DAOP) (reviewed in [1–3]). Most of the ARS generate Ap₄A from aminoacyl adenylate enzyme complex and ATP, while some other are capable of producing Ap₃A from adenylate and ADP. Mammalian tryptophanyl-tRNA synthetase (WRS, E.C. 6.1.1.2) contrary to the majority of other ARS cannot produce Ap₄A, but synthesises readily the Ap₃A [4]. This peculiar property of WRS is accompanied by another unusual feature, that is, inducibility by IFNs [5,6]. Human WRS gene possesses regulatory elements GAS and ISRE [7] typical for IFN-inducible genes (reviewed in [8,9]). Furthermore, these two independent observations are related since it was shown that WRS induction by IFNs was associated with Ap₃A accumulation in IFN-treated cells while the Ap₄A concentration was not affected [10]. Several hypotheses have already been suggested to ex-

plain the role of WRS in IFN signalling pathway [11], including those where the major role of WRS response to IFNs was ascribed to the ability of WRS to produce Ap₃A, not Ap₄A rather than to its main function, tryptophanyl-tRNA formation. It was suggested [11] that Ap₃A/Ap₄A ratio may serve for IFN-induced signal transduction. Since IFNs possess along with other activities a strong antiproliferative capacity, one of the consequences of the above mentioned hypothesis is that cell status may be somehow associated with intracellular Ap₃A/Ap₄A ratio.

To test this experimentally, we have explored the advantages of a human promyelocytic cell line HL60 capable to undergo either differentiation to macrophage-like cells induced by phorbol esters like TPA [12], or apoptosis provoked by an inhibitor of DNA topoisomerase activity, VP16 [13]. Here, we have compared the Ap₃A and Ap₄A content in HL60 cells at four different cultivation conditions: uninduced and induced by TPA, VP16, or IFN α . The entirely opposite effect observed in differentiating and apoptotic cells with regard to the Ap₃A/Ap₄A ratio is fully consistent with the idea of involvement of Ap₃A/Ap₄A ratio in cell signalling pathways.

2. Materials and methods

2.1. Cell labelling and DAOP detection

The HL60 human myeloid leukaemia cell line was grown in RPMI medium supplemented with 10% foetal calf serum. Before addition of IFN α (1000 IU/ml), or TPA (150 ng/ml) or VP16 (20 μ g/ml) cells were grown 24 h with [³²P]orthophosphate (50 μ Ci/ml, 5000 Ci/mmol). For extraction of DAOP, 10% ice-cold perchloric acid was mixed with 2.0–2.5 $\times 10^7$ of cells. After 10 min on ice with gentle shaking the mixture was centrifuged 10 min at 1000 $\times g$ and the supernatant was neutralised with 5 M K₂CO₃. The pellet was discarded and the neutralised extract was treated with *E. coli* alkaline phosphatase (1.25 μ g/ml in 25 mM Tris-HCl, pH 8.0 and 10 mM MgCl₂) 3 h at 37°C. After 4-fold dilution with water the extract was applied on a DEAE-cellulose column (0.8 \times 10 cm) and nucleotides were eluted with a linear 0.2–0.8 mM of NH₄HCO₃, pH 8.2 (12 ml/h). Thin-layer chromatography of DAOP was performed on Silufol (Kavalier, Czechoslovakia). For autoradiography, the dried plates were kept in contact with X-ray film (Kodak X-Omat AR) for 48 h. In order to estimate the amount of the labelled nucleotides, the corresponding areas were removed from plates and counted in the toluene scintillation liquid.

2.2. Induction of apoptosis and examination of DNA

The HL60 cells were incubated in the presence or absence of 20 μ g/ml of VP16. Cells were lysed by addition to the culture medium of 5 \times lysis buffer (2 M NaCl, 50 mM Tris-HCl, 10 mM Na EDTA, pH 8.1, 1% SDS, 0.25 mg/ml of proteinase K), and DNA was extracted by the salting-out method. Electrophoresis was carried out on 1% agarose gel containing 0.5 μ g/ml ethidium bromide.

2.3. Fluorescent microscopic identification of apoptotic cells

Suspensions of control and VP16-treated cells were centrifuged for 5 min at 1000 $\times g$, the pellet was resuspended in serum-free medium,

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Abbreviations: Ap₃A, P¹,P³-bis(5'-adenosyl)triphosphate; Ap₄A, P¹,P¹-bis(5'-adenosyl)tetrakisphosphate; ARS, aminoacyl-tRNA synthetases (E.C. 6.1.1); DAOP, diadenosine oligophosphates; PBS, 150 mM NaCl in 150 mM sodium phosphate buffer, pH 7.2; TPA, 12-O-tetradecanoylphorbol-13-acetate; VP16, Epipodophyllotoxin

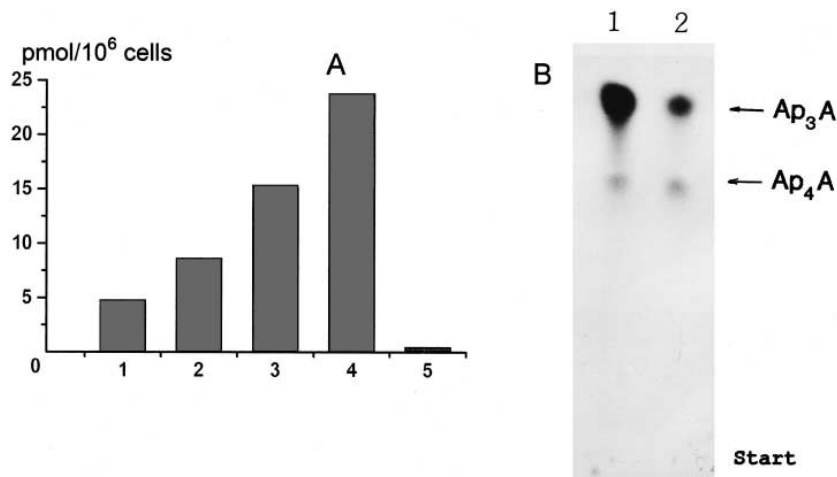


Fig. 1. DAOP content in IFN- and TPA-treated HL60 cells. A: Control (1); IFN: 15 h (2); 22 h (3); TPA: 48 h (4); Ap₄A content (5). B: TLC of labeled DAOP on Silufol plates. IFN: 22 h (1); control (2).

centrifuged once more and resuspended in PBS. Then, cell smears were prepared, dried, fixed with 70% ethanol, stained with 1 µg/ml of Hoechst 33258 (Sigma) in PBS, embedded in glycerol and examined under Leitz fluorescent microscope (Ob.X60) for the presence of apoptotic cells with fragmented pycnotic nuclei.

3. Results

3.1. DAOP synthesis in IFNα- and TPA-treated cultured human cells

The Ap₃A content in HL60 cells incubated with IFNα for 15 h and 22 h increased 1.8- and 3.2-fold, respectively, over the control (non-treated) level of 4.8 pmol/10⁶ of cells (Fig. 1). The level of Ap₄A was considerably lower (0.46 pmol/10⁶ of cells) and was not affected by IFN treatment of cells. These results are compatible with the data obtained earlier with non-induced human cell cultures [2].

TPA-treated HL60 cells exhibit intense adherence to plastic with prominent pseudopodia formation in parallel with a rapid profound loss of proliferative capacity. We measured the

Ap_nA concentration in untreated and TPA-treated cells. It appeared that after 48 h incubation with TPA the Ap₃A content significantly increased and reached the level of 24 pmol/10⁶ of cells. In control cells the level of Ap₃A was 5.2 pmol/10⁶ of cells. The Ap₄A content was insensitive to TPA treatment.

3.2. DAOP in HL60 cells undergoing to apoptosis

The treatment of HL60 cells with VP16 resulted in gradual accumulation of apoptotic cells characterised by fragmented nuclei with condensed chromatin as revealed by staining with a DNA-specific fluorochrom Hoechst 33258 (Fig. 2A). In control cultures apoptotic cells represented less than 0.5% of total cell population. Their proportion increased to 9% after 6 h and further to 35% at 24 h of VP16 treatment. DNA extracted from VP-treated HL60 cells showed a characteristic fragment size distribution after pulsed-field gel electrophoresis (Fig. 2B). The DNA fragments about 50 kbp in length were accumulated in response to VP16 treatment for 3 h. The second component of DNA fragmentation, the characteristic DNA

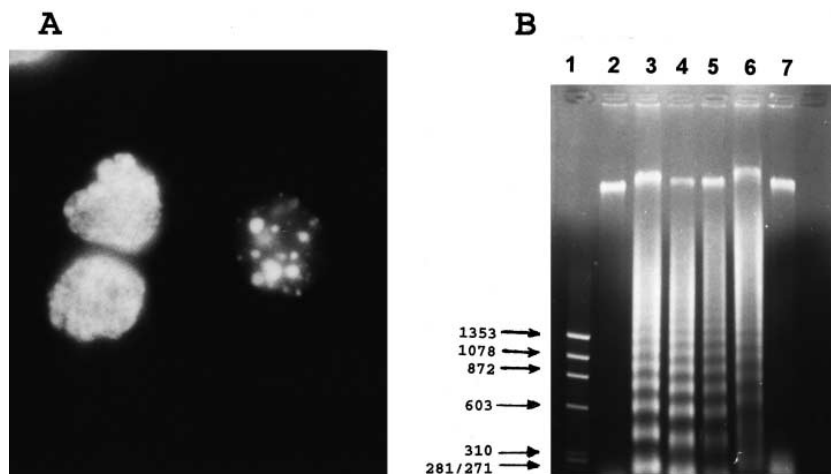


Fig. 2. VP16-induced apoptosis in HL60 cells. A: An apoptotic cell characterised by nuclear fragmentation and chromatin condensation and two normal cells. HL60 cells were treated with VP16 for 6 h. Fixed cells smear was stained with Hoechst 33258 and photographed. B: Agarose gel electrophoresis of total cellular DNA from HL60 cells showing the pattern typical for apoptosis. Lane 1, molecular weight markers (ϕX174 DNA cleaved with *Hae*III). DNA extracted from control HL60 cells at 0 time and after 10 h of cultivation (lane 7). DNA extracted from HL60 cells incubated with VP16 for 5, 10, 15 and 20 h (lanes 3–6, respectively).

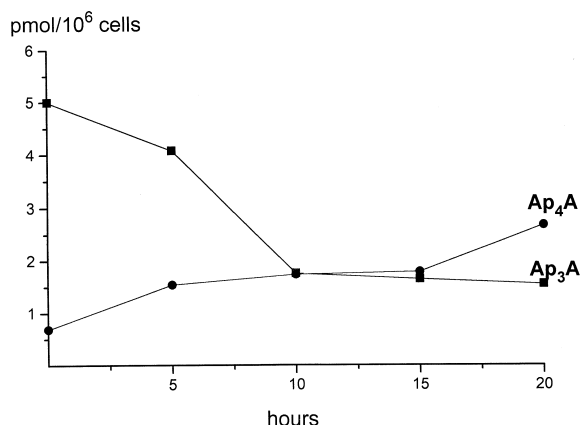


Fig. 3. Ap₃A and Ap₄A content in VP16-treated HL60 cells.

ladder of 200 bp (Fig. 2B) occurred when the cells became smaller after 10 h incubation with VP16. The full apoptosis was detected at 20 h of incubation with VP16.

The Ap₃A and Ap₄A concentrations in HL60 cells incubated with VP16 at various time intervals were measured. It appeared that the Ap₃A concentration tremendously decreased during the first 10 h of treatment with VP16; during the second 10 h of incubation with VP16 the level of Ap₃A didn't change significantly. At the same conditions the Ap₄A concentration reached 2.7 pmol/10⁶ of cells. Ap₄A was detected in control cells at the level of 0.7 pmol/10⁶ of cells.

4. Discussion

We have observed for the first time that apoptosis is associated with a dramatic inversion of the Ap₃A/Ap₄A ratio. In normally proliferating HL60 cells this molar ratio is roughly 7 while in the highly apoptotic cell culture it drops to about 0.5 (Fig. 3). This inversion is due to elevation of Ap₄A concentration in apoptotic cells and drop of Ap₃A level. The entirely opposite effect was observed when HL60 cells undergo differentiation (Fig. 1): the Ap₄A concentration remained constant whereas the Ap₃A level was manifold elevated. The analysis of data available in literature implies that (i) active cell proliferation is usually associated with the elevation of Ap₄A level while the opposite states such as quiescence and differentiation have no significant effect on the Ap₄A concentration. It is also interesting to note that in most available cases the intracellular concentration of Ap₃A moved in a direction opposite to that of Ap₄A. Consequently, it appears that Ap₃A/Ap₄A ratio rather than their concentrations taken separately is the most sensitive indicator of cell status. In this connection, it is necessary to stress that, as we have demonstrated, the Ap₃A/Ap₄A ratio shows polarly distinct behaviour in apoptotic versus differentiating HL60 cells. These data indicate a profound difference between the apoptosis and differentiation signalling in myelomonocytic cells, though the process of differentiation is usually accompanied with some degree of apoptosis [14]. Indeed, numerous observations have already shown that apoptosis and differentiation may be considered as alternative outcomes for proliferating leukaemic cells. Thus, the forced expression of the *bcl-2* gene not only prevents the apoptosis of leukaemic cells but also induces the transition from cell cycle to G₀ state typical for differentiation

[15]. Some inducers of differentiation like a derivative of vitamin D protect HL60 cells from apoptosis [16]. At the same time, some early effects of differentiation and apoptosis inducers, such as transient hyperexpression of *c-fos* and *c-jun* genes, are strikingly similar [17]. This is why the new data pertaining to the differences in cell signalling during apoptosis and differentiation are of considerable value. These data are helpful for understanding the mechanisms of these two alternative processes in leukaemic cells and are also of potential usefulness for the development of new chemotherapeutic approaches.

The potential signalling mechanisms relating the intracellular Ap₃A/Ap₄A ratio and cell status remain obscure. Several proteins were identified as possessing an Ap₄A binding ability [18–20]. We have detected in human cultured cells three proteins specific for the Ap₃A binding (A. Vartanian, unpublished observations). We assume that one of the most plausible ways to influence the cell behaviour via Ap₃A/Ap₄A ratio may rely on competition between binding of various DAOP to different proteins which may serve as signal transducers in bound or unbound state. Obviously, this speculation deserves further experimental verification.

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