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Opposite effects of cell differentiation and apoptosis on Ap_3A/Ap_4A 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 Ap_3A contrary to Ap_4A 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 Ap_3A concentration in HL60 cells induced by TPA, similar to the effect of IFN, while the Ap_4A concentration remained unchanged. On the contrary, in cells undergoing apoptosis induced by VP16, a topoisomerase II inhibitor, the Ap_3A concentration considerably decreased, while the Ap_4A concentration increased. These findings combined with earlier results suggest an involvement of the Ap_3A/Ap_4A ratio in signal transduction pathways controlling the cell status.

Key words: Human cell H60; Apoptosis; Macrophage differentiation; Interferon; Diadenosine oligophosphate; Phorbol ester; Signal transduction

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 Ap_4A from aminoacyl adenylate enzyme complex and ATP, while some other are capable of producing Ap_3A from adenylate and ADP. Mammalian tryptophanyl-tRNA synthetase (WRS, E.C. 6.1.1.2) contrary to the majority of other ARS cannot produce Ap_4A, but synthesises readily the Ap_3A [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 Ap_3A accumulation in IFN-treated cells while the Ap_4A concentration was not affected [10]. Several hypotheses have already been suggested to explain 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 Ap_3A, not Ap_4A rather then to its main function, tryptophanyl-tRNA formation. It was suggested [11] that Ap_3A/Ap_4A 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 Ap_3A/Ap_4A 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 Ap_3A and Ap_4A content in HL60 cells at four different cultivation conditions: uninduced and induced by TPA, VP16, or IFNα. The entirely opposite effect observed in differentiating and apoptotic cells with regard to the Ap_3A/Ap_4A ratio is fully consistent with the idea of involvement of Ap_3A/Ap_4A 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 IFNα (1000 IU/ml), or TPA (150 ng/ml) or VP16 (20 μg/ml) cells were grown 24 h with [^{32}P]orthophosphate (50 μCi/ml, 5000 Ci/mmol). For extraction of DAOP, 10% ice-cold perchloric acid was mixed with $2.0–2.5\times10^7$ 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 K_2CO_3. The pellet was discarded and the neutralised extract was treated with *E. coli* alkaline phosphatase (1.25 μg/ml in 25 mM Tris-HCl, pH 8.0 and 10 mM $MgCl_2$) 3 h at 37°C. After 4-fold dilution with water the extract was applied on a DEAE-cellulose column (0.8×10 cm) and nucleotides were eluted with a linear 0.2–0.8 mM of NH_4HCO_3, 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 μg/ml of VP16. Cells were lysed by addition to the culture medium of 5× 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 μg/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: Ap_3A, P^1,P^3-bis(5′-adenosyl)triphosphate; Ap_4A, P^1,P^4-bis(5′-adenosyl)tetraphosphate; 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

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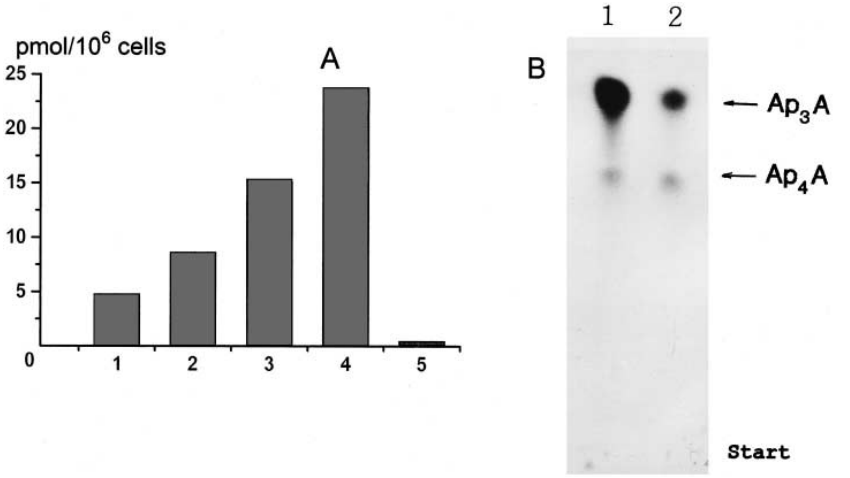

Fig. 1. DAOP content in IFN- and TPA-treated HL60 cells. A: Control (1); IFN: 15 h (2); 22 h (3); TPA: 48 h (4); Ap_4A content (5). B: TLC of labeled DAOP on Silufol plates. IFN: 22 h (1); control (2).

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 Ap_3A 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/10^6 of cells (Fig. 1). The level of Ap_4A was considerably lower (0.46 pmol/10^6 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 Ap_3A content significantly increased and reached the level of 24 pmol/10^6 of cells. In control cells the level of Ap_3A was 5.2 pmol/10^6 of cells. The Ap_4A 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

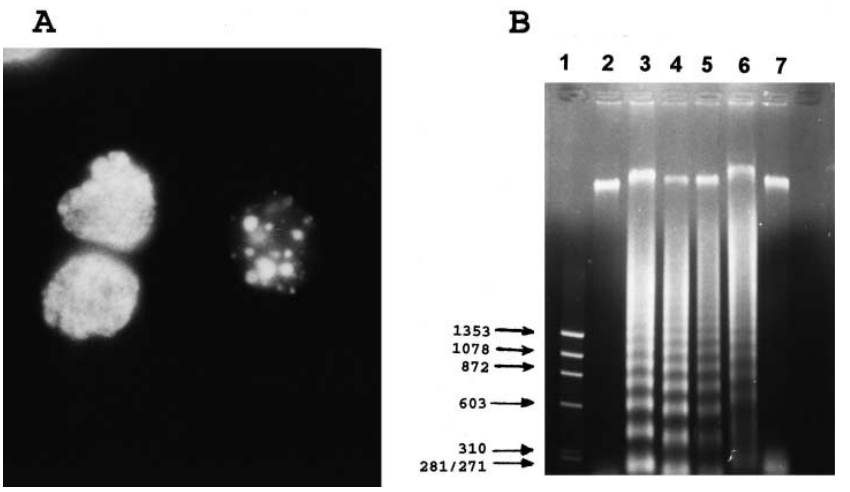

Fig. 2. VP16-induced apoptosis in HL60 cells. A: An apoptotic cell characterised by nuclear fragmentation and chromatin condensation and two normal cells. HL60 cells were treated with VP16 for 6 h. Fixed cells smear was stained with Hoechst 33258 and photographed. B: Agarose gel electrophoresis of total 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 O 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).

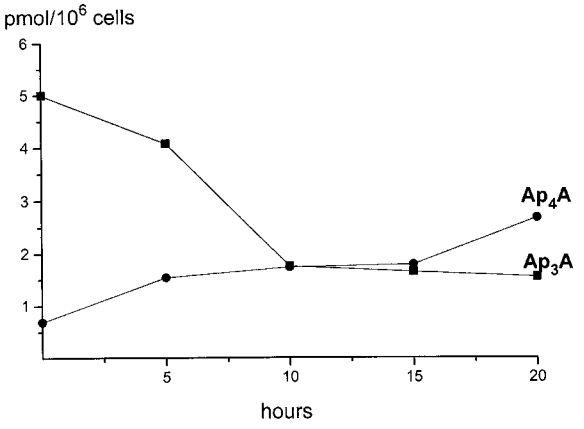

Fig. 3. Ap_3A and Ap_4A content in VP16-treated HL60 cells.

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 Ap_3A and Ap_4A concentrations in HL60 cells incubated with VP16 at various time intervals were measured. It appeared that the Ap_3A concentration tremendously decreased during the first 10 h of treatment with VP16; during the second 10 h of incubation with VP16 the level of Ap_3A didn't change significantly. At the same conditions the Ap_4A concentration reached 2.7 pmol/10^6 of cells. Ap_4A was detected in control cells at the level of 0.7 pmol/10^6 of cells.

4. Discussion

We have observed for the first time that apoptosis is associated with a dramatic inversion of the Ap_3A/Ap_4A 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 Ap_4A concentration in apoptotic cells and drop of Ap_3A level. The entirely opposite effect was observed when HL60 cells undergo differentiation (Fig. 1): the Ap_4A concentration remained constant whereas the Ap_3A level was manyfold elevated. The analysis of data available in literature implies that (i) active cell proliferation is usually associated with the elevation of Ap_4A level while the opposite states such as quiescence and differentiation have no significant effect on the Ap_4A concentration. It is also interesting to note that in most available cases the intracellular concentration of Ap_3A moved in a direction opposite to that of Ap_4A. Consequently, it appears that Ap_3A/Ap_4A 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 Ap_3A/Ap_4A 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 G_0 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 Ap_3A/Ap_4A ratio and cell status remain obscure. Several proteins were identified as possessing an Ap_4A binding ability [18–20]. We have detected in human cultured cells three proteins specific for the Ap_3A binding (A. Vartanian, unpublished observations). We assume that one of the most plausible ways to influence the cell behaviour via Ap_3A/Ap_4A 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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