Modulation of cell cycle progression and of antibody production in mouse hybridomas by a nucleotide analogue

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Abstract

The nucleotide analogue 9-[2-(phosphonomethoxy)ethyl]guanine (PMEG) has been identified as a powerful antiproliferative substance when acting on hybridoma cells. In the range of 10 nM to 100 nM concentrations this agent reduces cell growth rate, while its apoptosis-inducing activity is marginal. Marked induction of apoptosis can be observed at micromolar and higher order concentrations. In PMEG-supplemented media the cell cycle progression is perturbed, the flow-cytometric DNA profile shows a higher proportion of cells in the S and G₂/M phases of the cell cycle. Concomitantly with the reduction of the growth rate, the specific monoclonal antibody production rate may rise by 20–27%. Addition of PMEG at the end of the exponential phase of a batch culture results in an enhancement of the final monoclonal antibody concentration.

Abbreviations: MAb – monoclonal antibody; PMEG – 9-[2-(phosphonomethoxy)ethyl]guanine

Introduction

The production of MAbs is evidently not associated with the growth of hybridoma cells in a unique manner. Positively-, negatively-, or non-growth associated MAb production rates have been observed in various laboratories. The explanation of diverse findings by variation in culture conditions has been provided by the extended structural model of Suzuki et al. (1992). In our study of hybridoma growth in non-traditional unbalanced media we arrived at a conclusion that determinants different from those regulating growth and death of the cells control the intensity of MAb synthesis and secretion (Franěk and Šrámková, 1997).

Persistent cell proliferation and simultaneous significant death rate, occurring commonly in the stationary and decline phases of batch cultures, as well as in cultures run in the perfusion mode, are unfavorable for the production of secreted protein. A consider-

able portion of the nutrients is spent on synthesis of living cell mass, that is converted to dead cell mass within a short time period. The wasting of nutrients on synthesis of structural and informational macromolecules is particularly significant with hybridoma cells. Due to their somatic-hybrid nature hybridoma cells are hypotetraploid and possess thus an excessive load of DNA. It can be expected that the synthesis of the secreted MAbs would increase if hybridoma cell growth is inhibited without impairing cell viability.

Reduction of hybridoma growth by controlled feeding had, in our experience, a negative impact on the specific MAb production rate (Franěk, 1995; Franěk and Šrámková, 1997, 1998). Therefore, we decided to pursue an alternative approach based on the introduction of an antiproliferative molecular signal in a culture system saturated with substrates. Arresting the cell cycle while maintaining the cells in viable state has been recognized as a promising way to enhancing

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Figure 1. Inhibition of growth and induction of apoptosis in cultures of hybridoma ME-750 supplemented with PMEG at various concentrations. The data were obtained after 2 days in static culture. IG_{50} -PMEG concentration causing 50% inhibition of growth. AI_{50} -PMEG concentration at which the apoptotic index is equal to 50%. The experimental error of the data was ± 10 and $\pm 15\%$ for cell number and for apoptotic index, respectively (Franěk, 1995).

protein production by mammalian cells (Takahashi et al., 1994).

Substances endowed with antiproliferative capacity ('cytostatics') regularly induce apoptosis, depending on the concentration applied. Two criteria for selection of a suitable antiproliferative agent are relevant. First, the value of the lowest concentration exhibiting a significant inhibition of growth. Second, the ratio of the apoptosis-inducing concentration to the growth-inhibiting concentration. Among several acyclic nucleotide analogues the acyclic nucleoside phosphonate PMEG (Holý, 1994) was found to meet the first of above indicated criteria, being active at concentrations in the range of 10 to 100 nM, and was therefore selected for exploration of its effect on the cell cycle progression and on MAb production rate.

Materials and methods

Cell culture

Mouse hybridomas ME-750 and PVA-187 were cultured in DMEM/F 12/RPMI 1640 (2:1:1) medium supplemented with BME amino acids, 2.0 mM glutamine, 0.4 mM each of alanine, serine, asparagine and proline (Franěk and Šrámková, 1996), 15 mM HEPES, 2.0 g L⁻¹ sodium bicarbonate and with the iron-rich protein-free growth-promoting mixture containing 400 μ M ferric citrate (Franěk et al., 1992). All cultures were kept at 37 °C in a humidified atmosphere containing 5% CO₂. The static cultures were conducted in 25 cm² T-flasks. For estimation of growth inhibition, apoptosis and cell cycle profiles the cultures were inoculated at viable cell density $(400 \pm 50) \times 10^3$ mL⁻¹ and incubated for 2 days. Stirred cultures were carried out in spinner flasks.

Figure 2. Distribution of cell cycle phases in cultures of hybridoma ME-750 supplemented with PMEG at various concentrations. The data were obtained by DNA flow cytometry of cells harvested after 2 days in static culture.

Sample analyses

Viable cells and dead cells were counted in a hemocytometer using trypan blue exclusion test. The experimental error involved in the estimation of cell count and viability was $\pm 10\%$. The MAb concentration was determined by immunoturbidimetry (Fenge et al., 1991). Two parallels were set up for each sample evaluation. The experimental error associated with the estimation of MAb concentration was $\pm 5\%$.

The apoptotic index, i.e., the percentage of apoptotic cells relative to total cell density, was determined by microscopic counting of morphologically distinct shrunken cells with ruffled membrane, as described in detail before (Franěk, 1995). The concentration of glutamine was determined using TC L-glutamic acid kit with asparaginase (Boehringer Mannheim, Germany). For the determination of glucose the kit Oxochrom glucose (Lachema, Brno, Czech Republic) was employed.

Cell cycle profiles

For determination of the cell cycle profiles the cells ($5\text{--}10 \times 10^6 \text{ mL}^{-1}$) were permeabilized and stained

with propidium iodide using DNA Prep Reagent Kit (Coulter Immunology, Hialeah, FL) according to manufacturer's protocol. Twenty to 120 min after completing the staining procedure the samples were measured with EPICS Profile II flow cytometer (Coulter Immunology, Hialeah, FL) and data were analyzed by Multicycle software (Phoenix Flow Software Products, San Diego, CA). Percentage of cells in G_1 , S, and in G_2/M phases were evaluated.

Antiproliferative agent

PMEG, i.e. 9-[2-(phosphonomethoxy)ethyl]guanine, was synthesized as described before (Holý et al., 1989). Stock solution at 1 mM concentration was prepared in PBS and stored at $+4^\circ\text{C}$. The solution was stable for several months.

Results

Relation of growth inhibition and apoptosis

With the aim at evaluating quantitatively the relationship between growth-inhibiting and apoptosis-inducing activities of PMEG an experiment was set

Figure 3. Growth and MAb production in stirred cultures of hybridoma PVA-187 upon supplementation with PMEG. The cultures were set up in 300-ml volumes placed in 1 l-spinner flasks. PMEG was added to one of the cultures on day 3, as indicated by arrow. The final concentration of PMEG was 50 nM. The culture was fed on days 2 to 5 by doses of 300 mg L⁻¹ glutamine. Open symbols – control; closed symbols – culture supplemented with PMEG.

up, in which ME-750 hybridoma cells were incubated for 2 days in the presence of various concentrations of PMEG. The inhibition of growth was expressed as the viable cell density relative to the viable cell density in the control without PMEG. The degree of apoptosis was represented by the apoptotic index (Figure 1).

The two sigmoid curves intersected at a value of approximately 30%. The concentrations representing the 50% changes of the parameters, i.e. IG₅₀ (PMEG concentration causing 50% inhibition of growth), and AI₅₀ (PMEG concentration at which the apoptotic index is equal to 50%), differed by almost one or-

der of magnitude. The data in Figure 1 show that in the concentration range up to 100 nM the growth-inhibiting activity of PMEG is not accompanied with any substantial increase of apoptosis.

The changes in the cell cycle phases under influence of PMEG were examined by DNA flow cytometry (Figure 2). The inhibition of growth by PMEG, observable at PMEG concentrations >10 nM (Figure 1), was accompanied with a shift in the flow-cytometric DNA profile towards a higher proportion of the S and/or G₂/M phases of the cell cycle, and, consequently, with a reduction of the G₁ phase fraction. The degree of the shift progressed with increasing PMEG concentration. While at 10 nM PMEG the G₁ phase fraction represented 90% of the value observed in the control culture, at 100 nM PMEG the G₁ phase fraction dropped to 55% of the value in the control culture (Figure 2). The flow-cytometric analysis of DNA content thus showed that any significant reduction of hybridoma growth rate by application of PMEG would be accompanied with an altered proportion of cells in individual cell cycle phases.

MAb production

The influence of growth inhibition by PMEG on the specific MAb production rate was first examined in cultures to which PMEG was added at inoculation and was present during the whole culture period. The specific MAb production rate was found to be higher by 27%, relative to control, in the presence of 100 nM PMEG (Table 1). However, the final MAb concentration was lower than in the control, due to lower final viable cell density. Therefore, in further experiments PMEG was added at concentrations in the range of 10 to 50 nM at later stages of culture progression, and, in one case, in two portions.

A pair of stirred batch cultures of ME-750 hybridoma were set up starting from a single inoculation suspension. PMEG was added to one of the spinner cultures in two portions (Table 2). The final MAb concentration was 58 mg L⁻¹ in the control, and 63 mg L⁻¹ with PMEG, respectively. Thus, in this simple mode of batch culture the enhancement of the final MAb concentration upon addition of PMEG was found to be marginal. On the other hand, notable difference between the specific MAb production rates was found at the early decline period, i.e., between days 3 and 5. While in the control the average q_{MAb} dropped in this period to 6.95 pg cell⁻¹ d⁻¹, in the culture with PMEG the average q_{MAb} value

8.70 compared well with the highest q_{MAb} values attained in the exponential phase. The longer persistence of high specific MAb production rate in the PMEG-supplemented culture, and the fact that the pool of substrates was identical in both the control and in the PMEG-supplemented culture, indicated that available substrates were preferentially utilized for the synthesis of the secreted antibody.

The drawback of the above reported experiment was the decline of the specific MAb production rate on days 5 to 6 (Table 2). We explain this fact by exhaustion of critical substrates, likely to occur in cultures run in the trivial batch mode. Therefore, in similar experiments carried out with the hybridoma PVA-187 the spinner cultures were fed with glutamine to ensure maximum energy supply for MAb synthesis (Franěk, 1995; Franěk and Šrámková, 1998). The data of a representative experiment of this series are illustrated in Figure 3. Upon addition of PMEG to one of the spinner cultures on day 3 the MAb concentration began to rise above the level of MAb concentration in the control culture. An analysis of the cell cycle profile of samples withdrawn at day 4 showed 58 and 71% of S + G₂/M phase fractions in the control and in the PMEG-supplemented culture, respectively. These data are in agreement with the general trend of increase of the S + G₂/M phase proportion accompanying the addition of PMEG.

The experiment revealed a delayed decline of viable cell density in the PMEG-supplemented culture on days 6 and 7 (see also Table 2, days 5 and 6). The reason for this favorable persistence of viability is not obvious. More data and more detailed analyses are needed to understand this feature of PMEG activity.

Discussion

In the search for antiproliferative drugs analogues of nucleic acid components belong to one of the most promising classes of substances. Efficient cytostatics, such as 6-thioguanine, 6-mercaptopurine or 5-fluorouracil are known to be incorporated both to DNA and to RNA, after intracellular activation, and, in addition, might inhibit activities of some enzymes (Pizzorno et al., 1997). Their complex mode of action, that is advantageous for therapeutic purposes, represents a disadvantage in production cultures, because damage of RNA inevitably impairs the synthesis of secreted proteins.

Table 1. Specific MAb production rate in hybridoma ME-750 cultures carried out in the presence of PMEG. Parameters on day 3 in static cultures set up in 25 cm² flasks are given. Inoculation density was $410 \times 10^3 \text{ mL}^{-1}$

PMEG nM	Viable cell density $\times 10^{-3} \text{ mL}^{-1}$	Viability (%)	MAb mg L^{-1}	q_{MAb} $\text{pg cell}^{-1} \text{ d}^{-1}$	rel. %
0 (Control)	1590	83	43	10.7	100
10	1560	84	43	10.9	102
100	960	77	39	13.6	127

q_{MAb} – Specific MAb production rate.

Table 2. Specific MAb production rate upon addition of PMEG to a grown culture of hybridoma ME-750. Batch cultures were set up in 120-ml volumes placed in 250-ml spinner flasks

Day of culture	Spinner I					Spinner II				
	PMEG nM	$N_v \times 10^{-3}$ mL^{-1}	Viability (%)	MAb mg L^{-1}	q_{MAb} pg $\text{cell}^{-1} \text{ d}^{-1}$	PMEG nM	$N_v \times 10^{-3}$ mL^{-1}	Viability (%)	MAb mg L^{-1}	q_{MAb} pg $\text{cell}^{-1} \text{ d}^{-1}$
0	0	470	90	12		0	470	90	12	
1	0	750	95	17	8.2	0	750	95	17	8.2
2	0	1310	94	26	8.7	10	1300	93	26	8.8
3	0	1570	86	38	8.3	30	1480	86	38	8.6
4	0	1260	70	48	7.1	30	1210	68	50	8.9
5	0	810	45	55	6.8	30	920	52	59	8.5
6	0	580	31	58	4.3	30	740	42	63	4.8

N_v – Viable cell density; q_{MAb} – Specific MAb production rate.

The acyclic nucleotide analogs have been designed with a specific aim to obtain substances resistant to most catabolic reactions in the target system (Holý, 1994). Among several N-(2-phosphonmethoxyethyl) derivatives, the guanine derivative PMEG was found superior to the adenine derivative, and to other derivatives, in the cytostatic efficiency (Veselý et al., 1990). PMEG belongs to agents that damage DNA during replication, i.e. it perturbs cellular processes in the S phase of the cell cycle. Its putative diphosphate derivative, PMEGpp, exhibits inhibitory activity toward cellular DNA polymerases α , δ and ϵ , and terminates DNA chain (Štětina et al., 1993; Kramata et al., 1996; Pisarev et al., 1997). *In vivo* antitumour activity of PMEG has been reported (Rose et al., 1990). Because the mode of action of PMEG is perturbation of DNA synthesis, slow-down in the rate of progression through S and G₂/M phases can be expected. Our observation of the progressive increase of the fraction of S and G₂/M phases with increasing PMEG concentration are in agreement with the above assumption. It is

necessary to have in mind, that the distinction between the S and G₂/M phases might be impaired by the fact that the DNA content of cells in the late S phase and in the G₂ phase is hardly distinguishable. Therefore, in our present interpretations of the data we preferred to adhere to the rough division into G₁ and S + G₂/M phases.

It has been assumed that transient inhibition of DNA synthesis, caused by low concentrations of PMEG, will not be accompanied by inhibition of protein synthesis, and that transient unbalanced growth (Kung et al., 1990) will result in enhanced specific MAb production rate. The guanine derivative PMEG has attracted our interest because its antiproliferative activity is apparent at concentrations ~1000-fold lower than are the concentrations at which traditional antiproliferative agents, butyrate (Kruh, 1982), thymidine (Terada et al., 1997), or mimosine (Orren et al., 1997), are applied.

The favorable ratio of growth-inhibiting activity to apoptosis-inducing activity of this compound, as

shown in Figure 1, pointed to the possibility of a harmless application of PMEG in the concentration range of 10 to 100 nM. The shift in the flow-cytometric DNA profile towards a higher proportion of the S and G₂/M phases is consistent with the assumed perturbation of DNA replication.

The expected enhancement of the specific MAb production rate could be shown both in static cultures and in stirred cultures (Tables 1 and 2). However, for the application in the field of animal cell technology the final yield of MAb is of decisive importance. The batch experiments (Table 2 and Figure 3) have shown that the addition of small doses of PMEG to a highly grown hybridoma culture is a promising mode of practical utilization of this substance in MAb production and, possibly, for production of recombinant proteins by cultured animal cells.

Providing an antiproliferative signal to a production culture through addition of a small synthetic molecule is an alternative to the genetic approach that utilizes the capacities of recombinant technology. Controlled overexpression of physiological growth regulators, such as IRF-1 (Kirchhoff et al., 1996) or p21, p27 and P53175P (Fussenegger et al., 1997) led to elevation of model protein production. Eventhough the spectrum of capacities of the recombinant techniques for improvement of production lines is higher than the reduction of growth rate, we believe that further research on growth control by synthetic substances of antimetabolite character may prove to be a short and inexpensive way to increasing efficiency of production processes.

The present pilot study has answered some basic questions. Further investigation is needed to accumulate more data and to explore the possible favorable effects of PMEG application in the perfusion mode of culture, or in systems with immobilized cells.

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