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Nucleoside pools of acyclovir-treated herpes simplex type 1 infected cells

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Summary

Nucleoside pools of herpes simplex type 1 (HSV-1)-infected and uninfected African green monkey kidney (GMK) cells and human fetal lung fibroblasts (HL) have been analysed with high-performance liquid chromatography (HPLC). The only nucleosides found in measurable amounts were deoxythymidine (dThd) and adenosine (Ado). The dThd pool seemed to be greater in GMK cells than in HL cells. dThd was also the only nucleoside excreted into the medium. HSV-1 infection reduced the dThd concentration of GMK cells. Addition of acyclovir (ACV) to HSV-1-infected GMK cells inhibited virus replication. This resulted in a dThd concentration similar to that of uninfected GMK cells. dThd added to HSV-1-infected GMK and HL cells reduced the antiviral action of ACV but not that of phosphonoformic acid (PFA). ACV is known to be activated mainly by HSV-induced deoxythymidine kinase (dTK), an enzyme which utilizes dThd as a substrate, while the action of PFA is independent of dTK. The low antiviral activity of ACV in GMK cells as compared to HL cells may be explained by the presence of high amounts of dThd in GMK cells.

acyclovir; herpes simplex virus type 1; nucleoside pools; high-performance liquid chromatography

Introduction

Several antiviral compounds such as iododeoxyuridine (IdU), bromovinyldeoxyuridine (BVdU) and acyclovir (ACV) are activated by herpes simplex deoxythymidine kinase (dTK). IdU is also activated by cellular kinases. The in vitro action of ACV seems to be dependent on the cell strain used for virus cultivation [1–4]. ACV was found to exert less antiviral activity in African green monkey kidney (GMK) cells than in other cells tested [4,5]. To achieve a similar antiviral activity, the concentration of

ACV in GMK cells had to be 20–100 times higher than in HL cells [4]. The activities of IdU and BVdU were also found to be cell dependent, while the effect of phosphonoformic acid (PFA, foscarnet) was not [6]. Differences in the pool sizes of naturally occurring nucleosides and/or nucleotides, or differences in the quantity of HSV dTK [7,8] induced, have been suggested to account for this cell dependence of inhibition [3,4]. It has been shown that uninfected GMK cells contain higher levels of deoxythymidine (dThd) than uninfected HL cells [9]. Since dThd competes with ACV for dTK, the amount of phosphorylated ACV may be reduced [8]. HSV infection and ACV treatment may alter the cellular metabolism of nucleosides. To clarify the importance of naturally occurring nucleosides, we studied the nucleoside pools of HSV-infected and uninfected GMK and HL cells incubated in the presence or absence of antiviral substances.

Material and Methods

Cells

African green monkey kidney (GMK) cells and human fetal lung fibroblasts (HL) were maintained in Dulbecco's modified Eagle's medium with 2% fetal calf serum and antibiotics. The cells were assayed every month with the Hoechst 33258 staining method [10] and were found to be free of mycoplasma.

Virus

HSV-1 strain F 9004 was obtained from Sjukvårdsstyrelsen, Stockholm, Sweden. The virus stock used in the present study had a titer of 5×10^7 PFU/ml. The HSV plaque reduction assay has been described earlier [4].

Drugs

The nucleosides deoxythymidine (dThd), adenosine (Ado), deoxyadenosine, cytidine, deoxycytidine, guanosine, deoxyguanosine and uridine were purchased from Sigma (St. Louis, MO, U.S.A.). ACV was synthesized by Dr. Karin Eklind (Astra AB, Södertälje, Sweden) as described by Schaeffer et al [11]. PFA was obtained from Prof. Bo Öberg (Astra AB, Södertälje, Sweden).

Chromatographic procedure

The reversed-phase high-performance liquid chromatography (HPLC) was performed using a Waters HPLC apparatus (Waters Assoc., Milford, MA, U.S.A.). Column: analytical reversed phase 5 or 10 μm Rad-Pak C_{18} (8 mm ∅ 100 mm). Chromatographic conditions: 10 mM sodium acetate, pH 4.7:acetonitrile (95.0:5.0); flow-rate 2.5 ml/min; temperature: ambient. Preparative Sep-Pak C_{18} cartridges were purchased from Waters Assoc.

The peaks were identified in three ways: (1) retention times, (2) addition of known standard substances, (3) comparison of the ratios between absorbance at 254 nm and at 280 nm, as recommended by Brown et al. [12]. The dThd peak was further identified

by gas chromatography and mass spectrometry. The peaks were quantified by calculation of areas under the curves, which were compared with a standard curve of commercially available dThd (Sigma, St. Louis, MO, U.S.A.).

Sample preparation

Confluent GMK and HL cells were either infected with HSV-1 (multiplicity of infection (m.o.i.) = 4), mock-infected or HSV-1-infected and treated with ACV. The cells were subsequently maintained under serum-free conditions for 9 h. The cells were then extracted in 0.5 M $HClO_4$ (perchloric acid, PCA) without any prior wash. Cell extracts of 1–2 $\times$ 10^8 cells were pooled and centrifuged and the supernatant was neutralized with 4 M KOH and 0.4 M KH_2PO_4. After another centrifugation the supernatant was loaded onto a Sep-Pak C_{18} cartridge. The cartridge was washed once with 0.5 ml of water and twice with 0.5 ml of 2.5% methanol in water. The nucleosides were eluted from the cartridge with 2 $\times$ 0.5 ml of pure methanol. The nucleoside extract was then concentrated to dryness under reduced pressure. Finally, the samples were redissolved in HPLC buffer before injection into the HPLC apparatus. This procedure has been described [13]. All analyses were done in quadruplicate. A disadvantage with our cell preparation procedure is that some medium always remains with the cells at the time of extraction. Washing the cells so that all medium is removed prior to extraction would also remove the low molecular intracellular nucleosides [13]. Therefore, the discarded medium was analysed in parallel with the cells, but without PCA extraction.

Results

Influence of externally added deoxythymidine on the antiviral action of ACV and PFA

The amount of externally added deoxythymidine (dThd) that influenced the antiviral action of ACV and PFA was assayed in HSV-infected HL and GMK cells (Table 1). A dThd concentration of 1–100 μM decreased the antiviral action of ACV in the infected HL cells. For instance, the addition of 10 μM dThd gave a 50% plaque reduction value (PR_{50}) for ACV of 27 μM in HL cells compared to 1.5 μM without added dThd. This value is only slightly lower than the PR_{50} of 46 μM for ACV in HSV-1-infected GMK cells without added dThd. In HSV-1-infected GMK cells, a higher concentration of dThd was required than in HL cells to influence the HSV plaque reduction by ACV. Increasing concentrations of dThd did not substantially affect the plaque reduction curves for PFA.

Cellular nucleoside content of uninfected and HSV-1-infected HL and GMK cells with and without ACV

In HL and GMK cells, only dThd and/or adenosine (Ado) were found in clearly measurable amounts (Tables 2 and 3). The other nucleosides (deoxyadenosine, cytidine, deoxycytidine, guanosine, deoxyguanosine and uridine) were found in low or non-measurable amounts.

TABLE 1

The influence of various deoxythymidine concentrations on the antiviral activity of ACV and PFA as measured by plaque inhibition

	Deoxythymidine concentration (μM)			
	0	1	10	100
ACV				
HL	1.5[a]	7.0	27	>100
GMK	46	48	>100	>100
PFA				
HL	160	172	n.d.	132
GMK	187	145	n.d.	200

[a] The concentration of antiviral substance (in μM) that reduces the number of plaques by 50%.

dThd could be measured in uninfected GMK cells (Table 2). The addition of a high concentration of ACV (100 μM) to uninfected GMK cells increased the cellular dThd level. HSV-1-infected GMK cells without ACV exhibited a lower concentration of dThd than uninfected cells, and ACV treatment again gave an increased level of dThd. The dThd levels of the GMK medium varied in the same manner as the dThd concentration of the cells.

HL cells were analysed in the same manner as the GMK cells (Table 2). dThd could not be measured in uninfected HL cells or their medium. No dThd increases were measurable upon HSV infection or ACV treatment.

The Ado content of HL and GMK cells was measured in the same manner as dThd (Table 3). The HL cells had a higher level of Ado than the corresponding GMK cells in all treatment groups. No detectable level of Ado was found in the medium.

The kinetics of nucleoside pools in uninfected cells

Appreciable amounts of dThd were found in the medium of GMK cells (Table 2). It was therefore of interest to study the dThd concentrations with respect to time after

TABLE 2

The average concentration and standard deviation of deoxythymidine in HSV-infected and uninfected HL and GMK cells and in the discarded medium

		No ACV		100 μM ACV	
		Cells (pmol/10^6)	Medium (μM)	Cells (pmol/10^6)	Medium (μM)
Mock-infected	HL	<1	<0.05	<1	<0.05
	GMK	6.3 ± 0.8	0.3 + 0.1	13.4 ± 2.9	0.7 ± 0.1
HSV-1-infected[a]	HL	<1	<0.05	<1	<0.05
	GMK	<1	<0.05	23.6 ± 7.0	0.8 ± 0.3

[a] Multiplicity of infection: 4.0; cells harvested at 9 h post-infection.

medium change (Fig. 1). The medium of stationary GMK cells was discarded and serum-free medium (dThd and thymine-free) was added. At various times samples were taken from the medium and the dThd concentration was measured. The amount of dThd increased with time. The nucleoside dThd therefore seems to be produced by the GMK cells and released into the medium. After 9 days, the extracellular dThd concentration was found to be about 19 μM. The intracellular Ado concentrations of uninfected HL and GMK cells were also analysed at various times after medium change. The Ado levels increased with time in both HL and GMK cells (data not shown).

TABLE 3

The average concentration and standard deviation of adenosine in HSV-infected and uninfected HL and GMK cells and in the discarded medium

		No ACV		100 μM ACV	
		Cells (pmol/10^6)	Medium (μM)	Cells (pmol/10^6)	Medium (μM)
Mock-infected	HL	29.8 ± 8.3	<0.05	37.2 ± 9.2	<0.05
	GMK	18.2 ± 5.5	<0.05	9.8 ± 7.8	<0.05
HSV-1-infected[a]	HL	54.9 ± 6.9	<0.05	28.4 ± 9.5	<0.05
	GMK	5.5 ± 1.5	<0.05	4.0 ± 2.5	<0.05

[a] Multiplicity of infection: 4.0; cells harvested at 9 h post-infection.

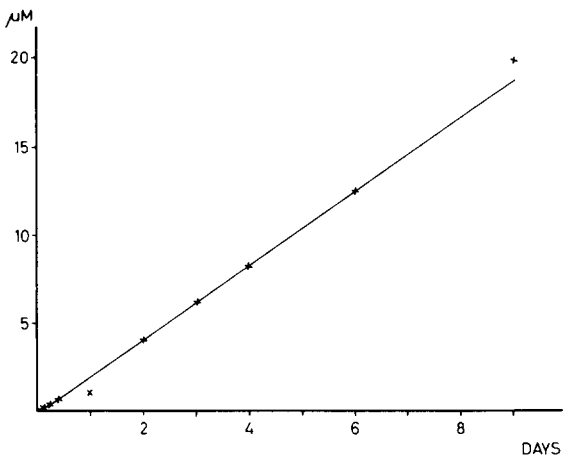

Fig. 1. The dThd concentration in the medium of GMK cells at different times after addition of medium to stationary cultures. Medium was removed from a tissue culture glass bottle containing approximately 50 × 10^6 GMK cells. The volume of the medium was 100 ml and the total cell volume approximately 0.25 ml.

Discussion

HSV-1 infection and ACV treatment may alter the cellular nucleoside metabolism. dThd and Ado were found in measurable amounts, while other nucleosides were undetectable or found in low concentrations. In all cases GMK cells contained higher levels of dThd than the corresponding HL cells. This supports the hypothesis that high intracellular dThd concentrations may be responsible for the low antiviral effect of ACV in HSV-infected GMK cells.

Addition of dThd to HL and GMK cells decreased the antiviral action of ACV. dThd was also added to PFA treated, HSV-1-infected HL and GMK cells. As expected, dThd did not influence the antiviral effect of PFA. This is also in agreement with the non-competitive action of PFA with its substrate [14].

It has previously been shown that addition of dThd and deoxycytidine (dCyd) to HSV-infected cells decreases the intracellular concentration of phosphorylated ACV [3]. Furthermore, dThd and dCyd are known to decrease the phosphorylation of ACV to ACV monophosphate by HSV-induced dTK [8]. Finally, Larsson and co-workers have shown that dThd addition reverses the antiviral effects of some nucleoside analogues, including ACV, as measured by the plaque reduction technique [15].

Since nucleosides penetrate the cell membrane, the cells cannot be washed to remove unwanted medium prior to extraction. Therefore a small volume of medium will remain with the cells in the extraction procedure. This will not affect the result if nucleosides are not present in the medium. dThd was measurable in both cells and medium, but Ado was not found in the medium. Simultaneous analysis of dThd in cells and medium provided an adequate estimate of the intracellular deoxythymidine pools. HSV-1-infected GMK cells contained approximately 10 times less dThd than uninfected GMK cells. Addition of 100 μM ACV to HSV-1-infected GMK cells increased the dThd concentration as compared to uninfected GMK cells. It is possible that HSV-1 infection increased both the production and the consumption of dThd. Addition of a high concentration of ACV would increase the dThd concentration because it inhibits HSV-1 DNA synthesis and decreases the phosphorylation rate of dThd through competition at the viral dTK level.

The concentration of Ado was generally higher in HL than in GMK cells. This appears to exclude the possibility that the nucleoside pools of GMK cells are generally elevated as compared to HL cells. The dThd pool therefore seems to be selectively increased in GMK cells. The dThd concentration of the medium of GMK cells increased with time and so did the concentration in the cells.

The results presented in this paper may serve as a basis for further studies on the importance of intracellular nucleoside pools. The nucleoside concentrations seem to be dependent on a number of variables, and conclusions based on quantitative measurements should therefore be cautious. For instance, the dThd content may vary between different tissues and species. Since a high cellular and medium dThd concentration may decrease the activity of an antiviral substance, the influence of medium changes and types of cells should be taken into consideration when evaluating antiviral potency of dTK-activated substances in vitro.

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