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RELEASING EFFECT OF THYMIDINE ON G1 PHASE ARREST OF ERYTHROLEUKEMIA CELLS CAUSED BY FUDR AND HMBA

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ABSTRACT *In vitro* experiments with synchronized MELC, FUDR plus HMBA produced a complete blockade of G1 phase cells. Thymidine at 0.1 m*M* released such a blockade and made the induction of differentiation by HMBA reappearing. Thymidine also released the arrest of G1 cells caused by FUDR + actinomycin D or sodium butyrate.

KEY WORDS thymidine; cell cycle; erythroleukemia cells; fluorodeoxyuridine; hexamethylene bisacetamide

The inductive effect of HMBA on differentiation of MELC is closely related to the prolongation of G1 phase and the initiation of accumulation of globin mRNA newly synthesized in G1 or early S phase[1]. Hydroxyurea or FUDR arrested elutriated G1 phase MELC at the G1/S boundary which was critical to the terminal differentiation of MELC[2]. The inhibitory action of FUDR on cell proliferation is dependent upon its blocking effect on thymidylate synthetase[3]. In this connection the question arises whether or not thymidine can reverse the blocking effect of FUDR on the G1 phase and the induction of differentiation of MELC caused by HMBA. The answer will provide more evidence for elucidating the role of G1 or S phase in the process of differentiation. In the present work the influence of thymidine on G1 block as well as the induction of terminal differentiation of MELC caused by FUDR and HMBA was investigated.

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Abbr. FUDR = 5-fluorodeoxyuridine; HMBA = hexamethylene bisacetamide; MELC = murine erythroleukemia cells

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MATERIALS AND METHODS

Cells MELC strain 745 A infected with Friend virus was provided by Charlotte Friend and the subclone DS_{19} has been maintained in continuous culture in this laboratory for several years[4]. The cells were grown in suspension in Eagle's basal medium with Earle's balanced salts supplemented with 15% fetal bovine serum. Cell number was determined with a model ZF Coulter Counter.

Cell cycle synchronization The Beckman centrifugal elutriation rotor was employed for fractionation of MELC and a logarithmic phase cells (in general not exceed 5×10^8) in 20 ml Hank's balanced salt solution without serum were placed into the spinning rotor. The elutriation was carried out at 20℃ by countercurrent centrifugation. The fraction of 250 ml was collected at each flow rate and the cells were recovered by 10-min centrifugation 1000 rpm at 4℃. An aliquot of 5×10^5 cells was stained with propidium iodide (50 μg/ml) containing RNase 30 μg/ml and the DNA content/cell was analyzed by flow microfluorometry (FMF; TPS-1 cell sortor, Coulter Electronic Co)[5]. In comparison with unsynchronized cell distribution curve in the control G2 phase cells possess DNA content (4 C) twice than the G1 cells (2 C), and the intermediate DNA content is defined as S phase cells[6]. Under the influence

of different compounds the FMF curve patterns at 4, 8, 12 h were analyzed in comparison with the control. Each compound was added to culture media prior to addition of cells and the experiments with the same dose were repeated at least twice. The pure G1 cells were chosen and put into normal medium as above-mentioned or into the medium with dialyzed serum which was dialyzed with PBS for 2 d in order to exclude thymidine and other related components in the serum.

Colony-forming efficiency MELC were placed into a semi-solid medium composed of 1.44% (w/v) methylcellulose (4000 centipoise, Fisher Scientific Co.) in Dulbecco's modified Eagle's medium (Gibco) with or without 5 m*M* HMBA[7]. Cells were suspended at 1×10^4 cells/ml in semi-solid medium and dispensed in 1 ml aliquot into 35-mm plastic Petri dishes (Falcon) with triplicates. The dishes were put in a humidified incubator at 37℃ with 5% CO_2. After 7 d the colonies containing several dozens to a few hundred cells were examined with an inverted stage microscope.

Cytological studies For liquid suspension the rapid scoring of differentiated cells was examined by a staining solution of 0.2% benzidine hydrochloride in 0.5% acetic acid under light microscope. The cells were also deposited on glass slides by means of a cytocentrifuge (Shandon Southern Instruments, Inc.) fixed in methanol and stained with benzidine-Wright-Giemsa.

Chemical compounds HMBA was certified reagent grade. FUDR was purchased from Cal Biochem. Co. (A grade) and thymidine was Sigma grade from Sigma Chem Co.

RESULTS

Influence of thymidine on the G1 arrest caused by FUDR and HMBA After centrifugal elutriation the synchronized G1 cells were collected and divided into 2 aliquots, one was cultured in normal medium and the other was in the medium containing dialyzed serum (DM). Both aliquots with or without HMBA (5 m*M*), FUDR (20 μ*M*), thymidine (0.001, 0.01 or 0.1 m*M*) or in combination of them were examined for the FMF curve patterns after every 4 h up to a total of 12 h (one cell cycle) and for the induction rate on d 5 after incubation as well. The results are shown in Table 1.

The control G1 cells cultured in normal medium or in DM had nearly the same FMF curve pattern without G1 arrest, no induction of differentiation was found either. HMBA itself caused G1 prolongation but no complete block, the induction rates were 95% and 78% in normal medium and DM, respectively. When HMBA was used in combination with FUDR, MELC were blocked completly at G1 phase and 5 d later the inductive activity of HMBA was

Tab 1. Influence of thymidine on G1 arrest and induction of differentiation caused by FUDR and HMBA. ±Prolongation without arrest, + Partial arrest, + + Marked arrest, + + + Complete arrest, H = HMBA (5 m*M*). F = FUDR (20 μ*M*), T = Thymidine.

	G1 arrest		Induction of differentiation (benzidine reactive%)	
	Normal medium	Medium with dialyzed serum	Normal medium	Medium with dialyzed serum
Control	−	−	0	0
HMBA 5 m*M*	±	±	95	78
FUDR 20 μ*M*	+	+ +	0	0
HMBA (5 m*M*) + FUDR (20 μ*M*)	+ + +	+ + +	2	1
H + F + Thymidine 0.001 m*M*	+	+	35	21
H + F + T 0.01 m*M*	±	±	64	34
H + F + T 0.1 m*M*	−	−	89	85
Thymidine 0.1 m*M*		−		2

Tab 2. Influence of thymidine on G1 arrest and induction of differentiation caused by FUDR and actinomycin D or sodium butyrate

	G1 arrest (in normal medium)	Induction of differentiation (%)
Control	−	0
FUDR 20 μM	+	0
Actinomycin D 1.5 ng/ml	±	65
Sodium butyrate 1.5 mM	±	83
FUDR 20 μM + Act. D 1.5 ng/ml	+ + +	0
FUDR 20 μM + Sod. butyrate 1.5 mM	+ + +	0
FUDR 20 μM + Act. D 1.5 ng/ml + Thymidine 0.1 mM	−	52
FUDR 20 μM + Sod. butyrate 1.5 mM + Thymidine 0.1 mM	−	48

also abolished by FUDR. Under the same condition thymidine 0.1 mM released the blockade of FUDR + HMBA from G1 phase, the induction of differentiation by HMBA reappeared too. The less concentration of thymidine was used, the weaker releasing effect was seen. It seems that thymidine plays an important role in the G1 arrest and induction of diffentiation produced by HMBA + FUDR.

Influence of thymidine on the G1 arrest caused by FUDR and actinomycin D or sodium butyrate Experiment was conducted with actinomycin D (1.5 ng/ml), sodium butyrate (1.5 mM) instead of HMBA in normal medium culture, and the results are presented in Table 2.

Actinomycin D 1.5 ng/ml and sodium butyrate 1.5 mM exerted the similar effect as HMBA on G1 phase and induced the differentiation of MELC at 65% and 83%, respectively. When these 2 inducers were used in combination with FUDR 20 μM, complete G1 arrest was recorded and the inductive activity was lost too. After adding 0.1 mM thymidine to these combinations, the G1 arrest disappeared and the induction rate was recovered. It is likely that the releasing effect of thymidine is not only in the case of HMBA, but also for other kinds of inducers. Thus such action of thymidine seems to have a property of general significance.

The optimal concentration of thymidine for its releasing effect Thymidine 0.2, 0.5, 1 and 2 mM did not increase the releasing efficacy, neither recover the induction of differentiation (Table 3). Thymidine 1 and 2 mM even exhibited a blocking effect on G1 phase but no induction of differentiation. Under the present conditions 0.1 mM was proved to be the optimal concentration of thymidine for its releasing effect.

Clonogenic assay for viability of MELC after manipulation of different compounds MELC were cultured in the medium containing dialyzed serum with HMBA, FUDR, thymidine or their combination, 12 h later the cells were washed twice with fresh medium and put into the semi-solid cloning medium containing methylcellulose. The clonogenic efficiency was examined on d 8 & 9.

Table 4 shows that clonogenic efficiency in the control is equal to 38–49%. After culture with HMBA, FUDR or thymidine for 12 h, the cells were still alive, and the colony-forming percentages were similar to the control. Under the influence of FUDR with HMBA for 12 h the MEL cells were also alive, and the colony formation was 36–38%, but the size of colonies was generally smaller than 0.5×1.32 mm^2 (total square of a calculator = 1.32 mm^2). In combined use with thymidine 0.1 mM the clonogenic efficiency was slightly higher than those without thymidine. Moreover, the size of the majority of colonies was larger than 0.5×1.32 mm^2. It is suggested that the cells after culture with above-mentioned compounds the viability is nearly the same, but the proliferation capabilities are different, i e, in the group

Tab 3. The optimal concentration of thymidine for its releasing effect on G1 arrest and induction of differentiation of MELC. F = FUDR 20 μM, H = HMBA 5 mM, T = Thymidine

	G1 arrest (in dialyzed medium)	Induction of differentiation (%)
Control	−	1
HMBA 5 mM	±	87
FUDR 20 μM	+	0
FUDR (20 μM) + HMBA (5 mM)	+ + +	0
Thymidine 0.1 mM	−	3
0.2 mM	−	1
0.5 mM	±	1
1.0 mM	+	1
2.0 mM	+	0
F + H + T 0.1 mM	−	85
F + H + T 0.2 mM	±	1
F + H + T 0.5 mM	+	5
F + H + T 1.0 mM	+ +	1
F + H + T 2.0 mM	+ +	9

Tab 4. Clonogenic assay for viability of MEL cells after 12 h treatment of different compounds, when the cells were washed twice and put into the semi-solid cloning medium.

	Clonogenic efficiency (%) in dialyzed medium	
	8th day	9th day
Control	38	49
HMBA 5 mM	51	60
FUDR 20 μM	32	36
Thymidine 0.1 mM	54	69
FUDR (20 μM) + HMBA (5 mM)	36	38
HMBA (5 mM) + Thymidine (0.1 mM)	53	59
FUDR (20 μM) + Thymidine (0.1 mM)	63	71
FUDR (20 μM) + HMBA (5 mM) + Thymidine (0.1 mM)	55	61

with thymidine the number of cells in one colony is more than that in other groups.

DISCUSSION

It has been reported[2] that both the prolongation of G1 and the onset of accelerated globin mRNA synthesis in HMBA-treated MELC appear to be the consequence of a critical inducer mediated event which occurs during the preceding late G1 or early S phase. Cells synchronized in mid-S or late S/G2 and exposed to HMBA display a delay in the onset of both these features of induced differentiation suggesting that they are not induced to differentiate until they have traversed the full cell cycle including the critical G1/early S in the presence of the inducer. Brown and Schildkraut[8] reported that unifilar substitution of bromodeoxyuridine for thymidine in DNA during early S-phase inhibits the growth and differentiation of MEL cells subsequently exposed to dimethyl sulfoxide.

Our data show that FUDR can strengthen the G1 prolongation caused by HMBA and make G1 arrest, meanwhile HMBA-induced differentiation was abolished. It suggests that during the induction of differentiation DNA synthesis including thymidylate synthesis is quite necessary. The fact that thymidine released the G1 block caused by FUDR and HMBA, and recovered HMBA induced differentiation supports the importance of DNA synthesis in S phase. Since such phenomenon is also observed in the experiments with other inducers, eg, actinomycin D and sodium butyrate, it might be speculated that during the induction of differentiation S phase pertinent to DNA

synthesis is the prerequisite.

The clonogenic efficiency tests show that the inhibitory effect of FUDR and HMBA on cell proliferation is reversible and can be changed by thymidine, which is one of the important precursors of DNA synthesis. It suggests that the differentiation process of MELC can be regulated either by inducers or by nucleosides. Eventually such findings may be useful to give new leads against malignant transformation.

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胸腺嘧啶核苷对氟尿嘧啶核苷和六甲烯二乙酰胺引起红白血病细胞 G1 期停止的释放作用

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提要 以体外培养的同步化小鼠红白血病细胞进行试验，氟尿嘧啶核苷和六甲烯二乙酰胺(HMBA)合用能使细胞完全停滞于 G1 期。胸腺嘧啶核苷 0.1 mM 能释放 G1 细胞滞留，并使 HMBA 诱导细胞分化的作用重现。胸腺嘧啶核苷也能释放由于氟尿嘧啶核苷和放线菌素D或丁酸钠引起的 G1 细胞滞留。

关键词 胸腺嘧啶核苷；细胞周期；红白血病细胞；氟尿嘧啶核苷；六甲烯二乙酰胺

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