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CHANGES OF *c-myc* EXPRESSION IN B16 MELANOMA CELLS INDUCED BY 8-CHLOROADENOSINE-3′, 5′-MONOPHOSPHATE AND TIAZOFURIN

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Abstract

The aim of this study was to investigate the *in vitro* effects of 8-chloroadenosine 3′, 5′-monophosphate (8-Cl-cAMP) and tiazofurin (TR) on the expression of *c-myc* gene in B16/F10 and B16/C3 mouse melanoma cells. Exponentially growing cells were treated with 8-Cl-cAMP or TR (5μmol - 25μmol) for 6h and 24h. The level of *c-myc* expression, estimated by RT-PCR, did not significantly change in B16/F10 cells after treatment with 8-Cl-cAMP or TR. Similar results were obtained in B16/C3 cells after treatment with 8-Cl-cAMP. The level of *c-myc* expression has shown a significant increase in B16/C3 cells after treatment with TR. Further studies of these agents will lead to better understanding of molecular mechanisms of their action.

Introduction

Disseminated malignant melanoma is among tumours resistant to radiation and chemotherapy. Drug resistance is caused by a deregulation of process of cell death. A more complete understanding of molecular mechanisms involved in the control of cell survival and death could potentially lead to the discovery of new drugs with the ability to interfere with specific cellular targets and finally more efficient therapeutic approaches for the treatment of melanoma [1, 2].

8-Cl-cAMP and tiazofurin are novel antineoplastic agents with selective effects on different tumour cells. 8-Cl-cAMP is a site-specific cAMP analogue that selectively modulates two isoforms of cAMP-dependent protein kinase A (PKA-I and PKA-II) [3]. Tiazofurin (2-β-D-ribofuranosyl-thiazole-4-carboxamide) is an inhibitor of inosine 5′-monophosphate dehydrogenase (IMPDH) type II, a rate-limiting enzyme in the synthesis of GTP and dGTP [4].

Materials and Methods

Mouse melanoma cells, B16/F10 and B16/C3, were maintained as a monolayer culture in RPMI 1640 medium supplemented with 10 % fetal calf serum, under standard conditions (37°C, 5%CO_2).

Total cellular RNA was isolated from B16 melanoma cells ($1x10^6$) using Qiagen RNeasy Total RNA Preparation kit according to the manufacturer′s instructions. For cDNA synthesis 1 μg of total RNA was reverse transcribed in 5 x RT buffer in the presence of 0.5 μM Oligo$(dT)_{16}$, 50 μmol/L of each dNTPs, 0.4 U/μL RNase inhibitor (Perkin-Elmer), 5 U/μL murine leukemia virus reverse transcriptase (MuLV

RT) in a final volume of 10 μL. The reverse transcription reaction (RT) was performed at 42°C for 1h and stopped at 95°C for 5 min. For the amplification of samples in polymerase chain reaction (PCR), appropriate amounts of cDNA samples, representing 200 ng of total RNA were mixed with PCR buffer, 1.5 mM $MgCl_2$, 200 μmol/L of each dNTPs, 0.25 μmol/L of each c-myc and GAPDH primer, 0.05 U/μL Taq polimerase in a total volume of 25μL. The samples were denaturated at 94°C for 5 min. The amplification cycle parameters were 94°C for 30″, 62°C for 30″ and 72°C for 30″ (35 cycles) followed by 72°C for 5 min. PCR-amplified products were analyzed on a 2% agarose gel. Quantification was performed using a Multi-Analyst/PC Software Image Analysis System (BioRad, Gel Doc 1000). The relative mRNA level of *c-myc* gene was normalized to the corresponding GAPDH mRNA level in individual samples and the respective mRNA changes were determined relative to the untreated samples. Primer sequences for *c-myc* were: upstream 5' CAA GAG GCG AAC ACA CAA CGT CT and downstream 3' AAC TGT TCT CGT CGT TTC CGC AA. The size of PCR product was 218 bp.

Results and discussion

Previous results have shown that 8-Cl-cAMP and TR significantly inhibit B16/F10 and B16/C3 cell growth. The growth inhibition is connected with the changes in cell cycle distribution in B16/F10 cells. The growth inhibition detected in B16/C3 cells is related to the induction of apoptosis [5]. Considering previous results and literature data concerning gene expression connected to apoptosis and proliferation, the effects of 8-Cl-cAMP and TR on *c-myc* expression were examined. Literature data indicated that *c-myc* plays an important role in melanoma pathogenesis and may, therefore, provide a potential target for treatment [6].

Exponentially growing B16 mouse melanoma cells were treated with increasing concentrations of 8-Cl-cAMP or TR (5μmol/L, 10μmol/L, 25μmol/L) for 6h or 24h. In order to modify the method for the detection and quantification of low abundance *c-myc* proto-oncogene mRNA, simultaneously with abundant GAPDH mRNA, various experimental conditions for RT-PCR were tested and optimized. Optimal concentrations of "primers" were established for GAPDH (0.25 μmol/L) and *c-myc* (0.25 μmol/L). Aliquots of cDNAs representing 200ng of total cellular RNA were used in the assay to co-amplify *c-myc* and GAPDH mRNAs. To estimate changes of *c-myc* expression, densitometric analysis of the agarose gels were performed. The obtained results have shown that in B16/F10 cells, 6h or 24h after treatment with 8-Cl-cAMP the level of *c-myc* expression did not significantly change **(1A)**. Treatment of B16/C3 cells with 8-Cl-cAMP in the duration of 6h did not cause significant changes in *c-myc* expression. Twenty four hours after treatment with 8-Cl-cAMP, the level of *c-myc* expression did not significantly increase in B16/C3 cells treated with 5μmol/L 8-Cl-cAMP and 10μmol/L 8-Cl-cAMP, but *c-myc* expression did significantly increase in the samples treated with 25μmol/L 8-Cl-cAMP ($p<0.05$) **(1B)**. The expression of *c-myc* did not significantly change in B16/F10 mouse melanoma cells 6h or 24h after treatment with tiazofurin **(1C)**. In B16/C3 mouse melanoma cells only 24h after the

same treatment, the level of *c-myc* expression increased significantly in all analyzed samples **(1D)**.

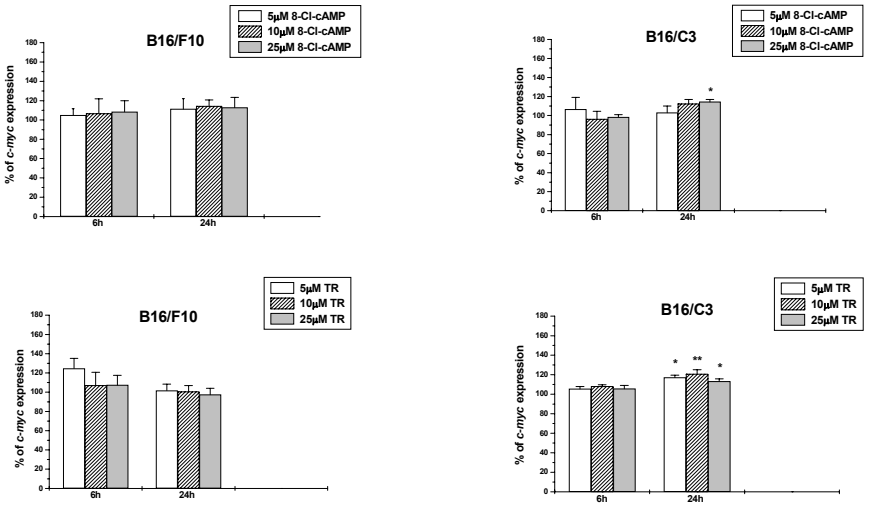

Figure 1. Level of *c-myc* mRNA expression after treatment of B16/F10 (A, B) or B16/C3 (C, D) melanoma cells with different concentrations of 8-Cl-cAMP or tiazofurin (TR) for either 6h or 24h. The densitometric analysis of *c-myc* normalized to GAPDH. Results are expressed as percent change relative to control levels set at 100%. Data represent the means ± S.E.M. from a minimum of 3 independent assays. (M-PCR marker, 1-control, 2-5μmol/L, 3-10μmol/L, 4-25μmol/L).

The duration of the mouse melanoma cells treatment with 8-Cl-cAMP or TR do not influence significant changes in the level of *c-myc* expression.

Conclusion

8-Cl-cAMP and tiazofurin did not change the level of *c-myc* expression in B16/F10 melanoma cells, as well as in B16/C3 cells 6h after treatment. Also, 8-Cl-cAMP (25μmol/L) and tiazofurin (5μmol/L, 10μmol/L, 25μmol/L) significantly increased *c-myc* expression, 24h after treatment.

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