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Cell division cycle control in embryonal and alveolar rhabdomyosarcomas

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Abstract

In this study, we investigated the mRNA level of several genes involved in cell cycle regulation in alveolar (ARMS) and embryonal rhabdomyosarcomas (ERMS). *p21^{Cip1}*, *Cyclin D1*, *Cyclin D2*, *Cyclin D3*, *CDK2*, and *CDK4* were evaluated by RT-PCR. All (13 out of 13) ERMS expressed the *p21^{Cip1}* gene compared with only 40% (4 out of 10) of the ARMS. Moreover, the amount of *p21^{Cip1}* mRNA was noticeably higher in the ERMS samples than in the positive ARMS specimens. p27^{Kip1} protein were analysed by immunohistochemical and immunoblotting. A noticeable difference was observed, in that ERMS had higher amounts of the cell cycle inhibitor compared with the ARMS. Finally, treatment of two rhabdomyosarcoma cell lines, RH-30 and RD, with butyrate, resulted in complete growth inhibition and in the upregulation of the p21^{Cip1} and p27^{Kip1} levels. Our results demonstrate that ERMS have a much higher level of p27^{Kip1} and p21^{Cip1} than the alveolar types, explaining, at least in part, the distinct features and outcomes (i.e. a poor prognosis of the alveolar type) of the two forms of this childhood solid cancer. Moreover, the data on butyrate-treated cell lines suggest that the two genes are potential novel therapeutic targets for the treatment of rhabdomyosarcomas.

Keywords: Rhabdomyosarcoma; Cell division cycle; Cell cycle controlling genes

1. Introduction

Rhabdomyosarcoma (RMS) is a highly malignant tumour of childhood arising from undifferentiated mesenchymal tissue which is similar to developing skeletal muscle.

Histologically, RMSs are classified into two main groups, embryonal (ERMS) and alveolar (ARMS) rhabdomyosarcomas. ERMSs are more frequent, particularly in younger children [1,2]. Patients affected by ERMS have a better prognosis when compared with those with ARMS [3]. The alveolar form, which is less frequent, is characterised by small round cells held together by strands of collagen. The cellular architecture resembles the alveolar spaces of the lungs. ARMSs, observable in older children, are more likely to occur on the limbs, and are associated with a higher-stage disease and a poor prognosis [3–5].

Different cytogenetic and molecular changes are associated with RMS. ARMSs are characterised by chromosomal translocations, t(2;13) (q35; q14) or t(1; 13) (p36; q14) [6–9], which result in a fusion gene between the undisrupted PAX DNA binding domains and the transactivation domain of the *FKHR* gene [10]. RMS also show genome amplification in the form of double minutes and homogeneously staining regions on chromosome 12q13 [6–9]. This region, which is commonly amplified in other sarcomas [11,12], includes several potentially important genes, including *GLI*, *SAS*, *CDK4* and *MDM2* [11]. Amplification of the 2p24 region and *MYCN* gene has been reported as well [13]. Using a cDNA microarray approach, the expression profile of an ARMS cell line, compared with a control cell line, has recently been reported [14]. 37 out of 1238

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genes screened were demonstrated to be upregulated and, among these genes, *CDK4*, *PAX-FKHR* and *MYCN* were identified, thus confirming the molecular observations described above [14].

Other genetic alterations that are relatively common in RMSs include: (i) the disruption of the imprinted gene cluster at chromosome region 11p15.5 [15,16]; (ii) *TP53* mutations [17,18], and (iii) loss of $p16^{INK4A}$ function by homozygous deletions, which have been described by us [19] and others [20].

At present, partly due to the rarity of RMS, very few studies have been carried out to investigate alterations in the expression profile of cell cycle genes in RMS, although their importance has been definitely demonstrated in a large variety of malignancies. Two major alterations in the cell cycle regulators have so far been observed in human cancers. One is inactivation of the $p16^{INK4A}$ gene (formally defined as the *CDKN2A* gene) and the other is reduced $p27^{Kip1}$ protein levels. Since *CDKN2A* gene transcription results in two independent mature mRNAs, its complete loss of function results in the absence of two distinct proteins, namely $p16^{INK4A}$ and $p14^{arf}$. $p16^{INK4A}$ controls the activity of two G1 cyclin-dependent kinases (CDKs) (i.e. CDK4 and CDK6), and thus its lack causes inappropriate pRB phosphorylation and an accelerated S phase entry [21]. $p14^{arf}$ protein, on the other hand, modulates the function of p53 (via the MDM2 protein) [21]. Thereby, loss of *CDKN2A* function impairs both the pRB and p53 pathways. In a previous study, we investigated the structure of the *CDKN2A* gene in rhabdomyosarcomas and reported its homozygous deletion in both cell lines and primary tumours [19].

$p27^{Kip1}$ inhibits both G1 and S CDKs (CDK4, CDK6 and CDK2) and thus, its cellular content controls not only the entry into S phase, but also progression through the other cell cycle phases. Tumours with low levels of $p27^{Kip1}$ have a poorer outcome when compared with neoplasias of the same stage, but with a higher content of the CDK inhibitor [22]. This finding has been demonstrated in several different human cancers including breast [23], colon [24], lung [25] and gastric tumours [26]. It should be underlined that $p27^{Kip1}$ cellular levels are regulated almost exclusively at the post-synthetic level and particularly by the rate of removal of the protein [27].

As described above, the development of RMS seems to be related to alterations in the normal skeletal muscle maturation. Since CDK inhibitors control the interplay of proliferation/differentiation, an analysis of their level appears particularly relevant to shed light on RMS development. However, few (if any) such investigations have been carried out. Therefore, we investigated the expression of cell cycle related genes in RMS tissue specimens. We also determined the level of $p27^{Kip1}$ by means of immunohistochemistry and Western blotting. Finally, we studied the possibility of modulating the levels of CDK inhibitors in RMS cell lines by using butyric acid, which is considered a promising molecule for cancer therapy.

2. Materials and methods

2.1. Tumour samples

Tumour biopsies were obtained at diagnosis from patients enrolled in the Italian national trial for the diagnosis and treatment of RMS. Samples were immediately frozen in liquid nitrogen and stored at −80 °C until use. Histological diagnosis was confirmed by the central review panel of pathologists, as per protocol. In order to ensure that the tumour samples contained a sufficient proportion of malignant cells, only samples which contained more than 90% of tumour cells were used in the present study. This ruled out the possibility that the results obtained were due to normal cells being present in the analysed specimens. All ARMS samples showed a *PAX/FKHR* fusion gene (data not shown).

2.2. Cell lines and treatments

Two human RMS cell lines, RH-30 and RD, were purchased from the American Type Culture Collection and grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). Butyrate was obtained from Sigma Chemical Company, St. Louis, MO, USA. 1 and 2 mM dilutions in distilled water were used. Rhabdomyosarcoma cells were plated at a low density (3000–4000 cells/cm^2) in order to avoid possible cell-to-cell contact inhibition. The cell lines were grown for 24 h before treatment with butyric acid. At the desired times, cells were collected, counted and pelleted as reported in Refs. [28,29].

In some experiments, the cell lines were treated concomitantly with the protein synthesis inhibitor cycloheximide (36 μM) and with (or without) 2 mM butyrate. After 8 h, cells were collected and total RNA was prepared as reported in Ref. [30].

2.3. Antibodies and reagents

Monoclonal antibodies against $p27^{Kip1}$ were from Transduction Laboratories (Lexington, UK). Monoclonal antisera towards $p21^{Cip1}$ were from PharMingen, San Diego, CA, USA, while rabbit polyclonal antibodies towards cyclins A, cyclin E and CDK2 were from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA. All other reagents for immunoblotting have been described in detail elsewhere in Refs. [28,29].

2.4. Reverse transcriptase polymerase chain reaction PCR (RT-PCR)

RT-PCR analysis was performed using the StrataScript RT-PCR Kit (Stratagene, La Jolla, CA, USA). Briefly, 2.5 μg of total RNA, prepared as reported by Iolascon and colleagues [31], were reverse-transcribed using StrataScript RNAase H^- reverse transcriptase (25 U) and oligo(dT) primer (150 ng) in a final volume of 25 μl. cDNA samples were diluted ten-fold in a PCR reaction assay to a volume of 50 μl containing, in addition to the DNA template, 30 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μg of each primer, 0.2 mM of each nucleotide and 1 unit of Taq DNA polymerase.

The expression of the *CDK2*, *CDK4* and *p21^{Cip1}* genes was determined by a co-amplification method using as an internal standard glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*) mRNA.

The following primers were used for the PCR reaction: *G3PDH*, forward 5′-GGTATCGTGGAAGGACTCATGAC-3′ and reverse 5′-ATGCCAGTGAGCTT-CCCGTCAGC-3′; *CDK2*, forward 5′-TTGACAAGAGCGAGAGGTATACTG-3′ and reverse 5′-AGATAGCTCTTGATGAGGGGAAG-3′, *CDK4*, forward 5′-AGATCAAGGTAACCCTGGTGTTT-3′ and reverse 5′ TCGACGAAACATTT-CTGCAA-3′; *p21^{Cip1}*, forward 5′-GGAAGGAAGGCTGGAAG-3′ and reverse 5′-CCGTTTTCGACCCTGAGAG-3′. Cycling conditions were: one cycle at 94 °C for 5 min, 30 cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and 1 cycle at 72 °C for 7 min.

The expression of members of cyclin D gene family (*cyclins D1*, *D2* and *D3*) was determined by a competitive RT-PCR assay as reported in Ref. [30].

Before amplification with each specific primer pair, an aliquot of the cDNA preparation was amplified using the *G3PDH* primers to determine the integrity of the generated cDNA. Moreover, we used five different cDNA concentrations to assure that signals (both of *G3PDH* and of the analysed gene) were proportional to the input mRNA. These controls are important for comparisons between the samples because they ensure that equivalent amounts of RNA are amplified. Finally, each experiment was performed at least in triplicate and, in most cases, four times.

Aliquots of PCR reactions were separated and analysed by electrophoresis on 2% (w/v) agarose gels or non-denaturing 8% (w/v) polyacrylamide gels (acrylamide/bisacrylamide, 29/1). In the latter case, the amplified products were detected using the silver nitrate staining method [31]. In several cases, the RT-PCR products were recovered from the gels and sequenced as reported in Ref. [31]: in all cases, the sequence of the amplified products corresponded to that reported in the literature.

2.5. Immunoblotting, immunoprecipitation and kinase assays

The preparation of extracts from the rhabdomyosarcoma specimens and cell line pellets has been described in Ref. [31]. Approximately 40–80 μg of proteins were resolved by 15% w/v sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis [28,29]. The proteins were transferred from the gel to a nitrocellulose membrane and western blotting performed as described in Refs. [28,29]. Immunoprecipitation experiments were carried out as reported in Ref. [29]. CDK2 activity was assayed using the immunoprecipitate with histone H1 as the phosphate acceptor.

2.6. Immunohistochemistry

Analyses were performed on histological slides from formalin-fixed and paraffin-embedded tumour tissues. The deparaffinised sections were treated with 3% v/v hydrogen peroxide for 15 min in order to quench the endogenous peroxidase activity. Thereafter, the sections were microwaved in 10 mM citrate buffer, pH 6, for 5–10 min, to retrieve the antigenicity. Monoclonal antibodies against $p27^{Kip1}$ were from Transduction Laboratories. After an overnight incubation at 4 °C with the primary antibody diluted 1:50, a biotinylated secondary anti-mouse antibody was applied at a 1:100 dilution for 30 min, followed by detection of the avidin-biotin-peroxidase complex (Vector Laboratories, Inc. Burlingame). For the immunostaining, the reaction was developed by 3-amino-9-ethylcarbazole and the sections were lightly counterstained with a haematoxylin (DAKO) [32]. The percentage of positive and negative cells was established as reported in Ref. [32].

2.7. Statistical analysis

The prevalence of a specific mRNA expression in the ARMS and ERMS samples was compared by the Fisher's exact test. A P value less than 0.05 was considered statistically significant.

3. Results

A total of 23 different RMS specimens (10 ARMS and 13 ERMS) were studied by semi-quantitative RT-PCR for the expression of the different genes controlling the cell cycle and cellular differentiation. The genes chosen were those for which the mRNA content has been reported to strictly parallel the protein level, namely *p21^{Cip1}*, *Cyclin D1*, *Cyclin D2* and *Cyclin D3*, *CDK4* and *CDK2*. On the other hand, since $p27^{Kip1}$ is regulated at a post-transcriptional level [22–27], the content of this CDK inhibitor was investigated in 13

RMS by immunohistochemistry and in 5 specimens by Western blotting. In some, but not all, of the cases the specimens studied by immunohistochemistry corresponded to those analysed by RT-PCR. This difference is due to the rarity of RMS and to the difficulty of obtaining sufficient tumour specimens for all of the analytical approaches employed.

3.1. Expression of cell division cycle-related genes in the primary specimens of rhabdomyosarcomas

Fig. 1 shows examples of *p21^{Cip1}*, *Cyclin D, CDK4* and *CDK2* gene expression in the RMS specimens analysed by semiquantitative RT-PCR. Cyclin D (including *Cyclins D1*, *D2* and *D3*) mRNA was observed in several tumour samples with no significant differences between the ARMS and ERMS (Table 1). In general, the data obtained suggest that *Cyclins D1* and *D2* are more highly expressed than *Cyclin D3*. The gene encoding the *CDK4* protein was expressed in the majority of specimens belonging to the two different histological types of rhabdomyosarcomas. Conversely, the *CDK2* gene appears quite rarely transcribed and, more frequently in the embryonal than the alveolar forms.

p21^{Cip1} mRNA is detectable in all of the ERMS samples examined (13 out of 13) but in only 4 out of 10 ARMS specimens (Table 1) (100% v 40%, respectively; $P<0.05$). The negative samples were overamplified (up to 45 PCR cycles) in order to verify the complete absence of *p21^{Cip1}* mRNA. The results obtained confirmed the lack of the *p21^{Cip1}* transcript (data not shown). In addition, the mRNA content of *p21^{Cip1}* was much higher (from 2 to 3-fold) in all of the ERMS samples compared with the four positive ARMS specimens (Table 1).

3.2. p27^{Kip1} protein content in human RMS

Table 2 shows the p27^{Kip1} protein level in 13 RMS specimens (5 alveolar and 8 embryonal forms). Fig. 2 shows examples of p27^{Kip1} negative (panel A) and positive (panel B) specimens. The data obtained, which were

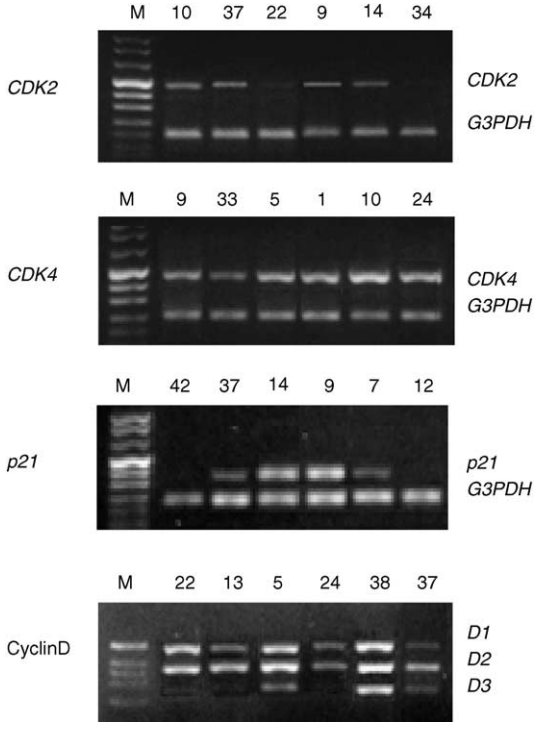

Fig. 1. Analysis of *CDK2*, *CDK4*, *p21^{Cip1}* and *Cyclin D1*, *Cyclin D2* and *Cyclin D3* mRNAs in human rhabdomyosarcoma specimens by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA (2.5 μg) was reverse transcribed cDNA samples were then diluted in a PCR reaction mixture and amplified by specific primers. Aliquots of the PCR reaction (5 μl) were separated on a 2% (w/v) agarose gel. *From the top to the bottom*: *CDK2*, *CDK4* and *p21^{Cip1}* and Cyclin *D1*, *D2* and *D3* analyses. *CDK2*, *CDK4* and *p21* cDNAs were coamplified with the glyceraldehyde 3-phosphate dehydrogenase (*G3PDH*) cDNA as an internal standard. Cyclin *D1*, *D2* and *D3* cDNAs were determined by a competitive RT-PCR assay [30]. The numbers reported represent the samples examined, M is the molecular weight standard.

Table 1
Expression of *p21^{Cip1}*, *CDK4*, *CDK2*, *Cyclin D1*, *Cyclin D2* and *Cyclin D3* genes in embryonal (ERMS) and alveolar (ARMS) rhabdomyosarcomas

Samples	*G3PDH*	*p21^{Cip1}*	*CDK4*	*CDK2*	*Cyclin D1*	*Cyclin D2*	*Cyclin D3*
ARMS							
1	++	–	++	–	–	–	+
7	++	+	+	–	+	+	–
10	++	–	+++	++	++	+	++
12	++	–	+	–	+	+	–
22	++	+	–	–	++	++	–
24	++	–	++	–	+	+	–
31	++	+	+	–	+	–	–
37	++	+	+	++	+	++	+
42	++	–	–	–	+	–	+
44	++	–	+	–	+	–	+
ERMS							
5	++	+++	++	–	++	++	+
9	++	+++	+	+	+	++	–
11	++	+++	++	+	+	+	–
13	++	+++	+	–	+	++	–
14	++	++	++	+	+	+	–
26	++	++	–	–	+	–	–
33	++	+++	+	+	++	+	–
34	++	++	–	–	+	+	–
38	++	+++	+	–	++	++	++
39	+	+++	+	–	–	–	+
40	++	++	+	+	+	–	–
41	+	+++	+	–	–	+	+
45	++	++	+	+	+	+	–

The symbol – represents the absence of mRNA, while the symbols +, ++ and +++ correspond to the relative values 1±0.4, 2±0.4 and 3±0.4 folds of the polymerase chain reaction (PCR) product, estimated by laser scanner analysis. These results were a mean of at least three independent experiments.

reported as the percentage of positive cells, indicate a high frequency, (4 out of 5 samples, 80%) of ARMS lacking the CDK inhibitor. The unique positive sample contained a very low percentage of positive cells (<10%). 5 out of 8 ERMS samples (>60%) were positive for $p27^{Kip1}$. In particular, 3 of these samples showed more than 30% positive cells (48, 51 and 32%, respectively). Although the small number of samples does not allow a statistical evaluation, the finding obtained strongly suggests there are differences in the $p27^{Kip1}$ expression in ARMS and ERMS.

The results obtained (Fig. 3) in an immunoblotting investigation using 5 different rhabdomyosarcoma samples (3 ARMS and 2 ERMS) confirmed that the alveolar form contains much lower amounts of $p27^{Kip1}$ when compared with the embryonal form. Equal loading of the proteins was confirmed by an actin analysis (Fig. 3) and by Red Ponceau colouration of nitrocellulose sheets after blotting (data not shown).

3.3. Effect of butyrate on $p21^{Cip1}$ and $p27^{Kip1}$ content in RMS cell lines

The data reported above indicate an inverse correlation between the aggressivity of RMS and the level of the two CDK inhibitors. This finding suggested that therapies aimed at increasing $p21^{Cip1}$ and $p27^{Kip1}$ content might be useful in the treatment of RMS. Therefore, we decided to evaluate the effect of butyrate, a promising non-toxic antiproliferative drug, on the growth of two rhabdomyosarcoma cell lines, RH-30 and RD.

Table 2
$p27^{Kip1}$ protein expression in embryonal (ERMS) and alveolar (ARMS) rhabdomyosarcomas

Samples	$p27^{Kip1}$ protein (% of positive cells)[a]
ARMS	
3	ND
6	ND
7	ND
10	<10
18	ND
ERMS	
2	48
5	51
8	ND
9	14
11	ND
13	ND
16	
17	13
32	

[a] Determined by immunohistochemical methodologies; ND, not detectable.

The butyrate concentrations (1 and 2 mM) are in the range of those generally used in experiments on cellular models (usually from 1 to 5 mM) [33,34] and are close to those reached during pharmacological trials [35,36].

As shown in Fig. 4, the addition of butyrate caused a complete inhibition of RH-30 cell proliferation (Fig. 4a) and a noticeable modification of cell morphology (Fig. 4b). Cytofluorometric analyses (Fig. 4c) demonstrated the accumulation of cells in the G1/S phase without any pre-G1 peak, thus ruling out the activation of the apoptotic process. When the levels of cell cycle proteins were analysed, we observed a clear increase of $p21^{Cip1}$ protein, a downregulation of cyclin A and an upregulation of cyclin E (Fig. 5a). This pattern is consistent with a block at G1/S transition. Importantly, $p27^{Kip1}$ protein levels were also upregulated (Fig. 5a). Subsequent immunoprecipitation experiments demonstrated that the two CDK inhibitors were mainly associated with CDK2 and, consequently, inhibited this kinase activity (Fig. 5b).

Identical results were obtained following treatment of RD cells with the short fatty acid. Butyrate induced cell growth arrest (Fig. 6a), clear morphological changes (data not shown) and an upregulation of the two CDK inhibitors (Fig. 6b). In order to further clarify the effect of butyrate, RD cells were incubated for 8 hours with or without 2mM butyrate in the presence of cycloheximide (36 μM). Then, total RNA was prepared and the content of *$p21^{Cip1}$* and *$p27^{Kip1}$* mRNA determined by RT-

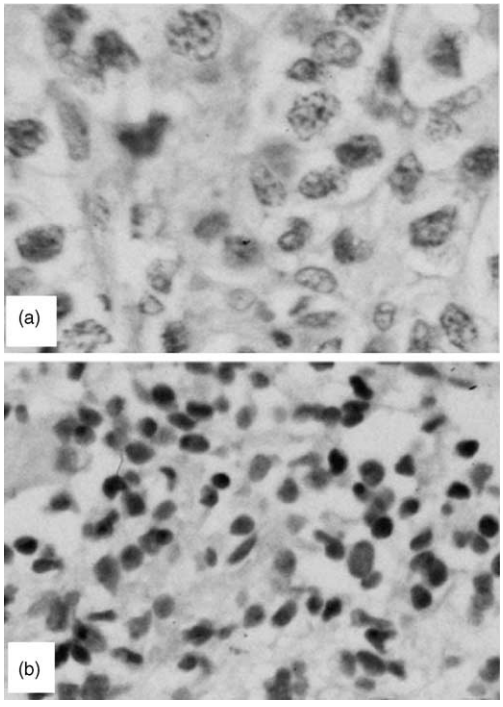

Fig. 2. Immunohistochemical analysis of $p27^{Kip1}$. Immunohistochemistry analysis of rhabdomyosarcoma specimens showing the absence of $p27^{Kip1}$ protein (panel a, sample 6) or the presence of the protein (panel b, sample 5).

PCR. The results reported in Fig. 7 show a large increase in *p21^{Cip1}* gene expression while the transcription of the *p27^{Kip1}* gene was almost unmodified.

Since these experiments were carried out in the presence of a protein synthesis inhibitor, they indicated that the *p21^{Cip1}* gene was a primary target of butyrate activity. Moreover, these results demonstrated for the first time that *p21^{Cip1}* gene might be manipulated by butyrate in rhabdomyosarcoma cell lines and, most intriguing, that the upregulation occurs independently of the promoter methylation status. On the other hand, the absence of a direct butyrate activity on *p27^{Kip1}* gene expression suggest that the increased level of this CKI is probably due to post-transcriptional mechanisms.

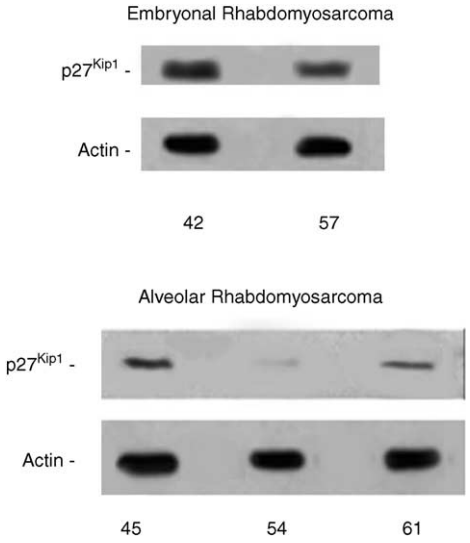

Fig. 3. Immunoblotting analysis of *p27^{Kip1}* protein in human alveolar and embryonal rhabdomyosarcoma samples. Specimens of rhabdomyosarcoma were analysed for the content of p27 and actin proteins.

4. Discussion

A wealth of studies have demonstrated a strong correlation between the level of p27^{Kip1} and the outcome of human cancer [23–26]. Low levels of the inhibitor correspond to poorer outcomes [23–26]. Mechanistically, the presence (or the absence) of the protein is not simply a consequence of an accelerated rate of proliferation, but it is due to a modulation of the protein degradation machinery [33]. The differences in the p27^{Kip1} level observed between ERMS and ARMS might therefore be important in relation to different prognosis of these two RMS types.

The removal of p27^{Kip1} is a complex process that involves an initial phosphorylation step on threonine

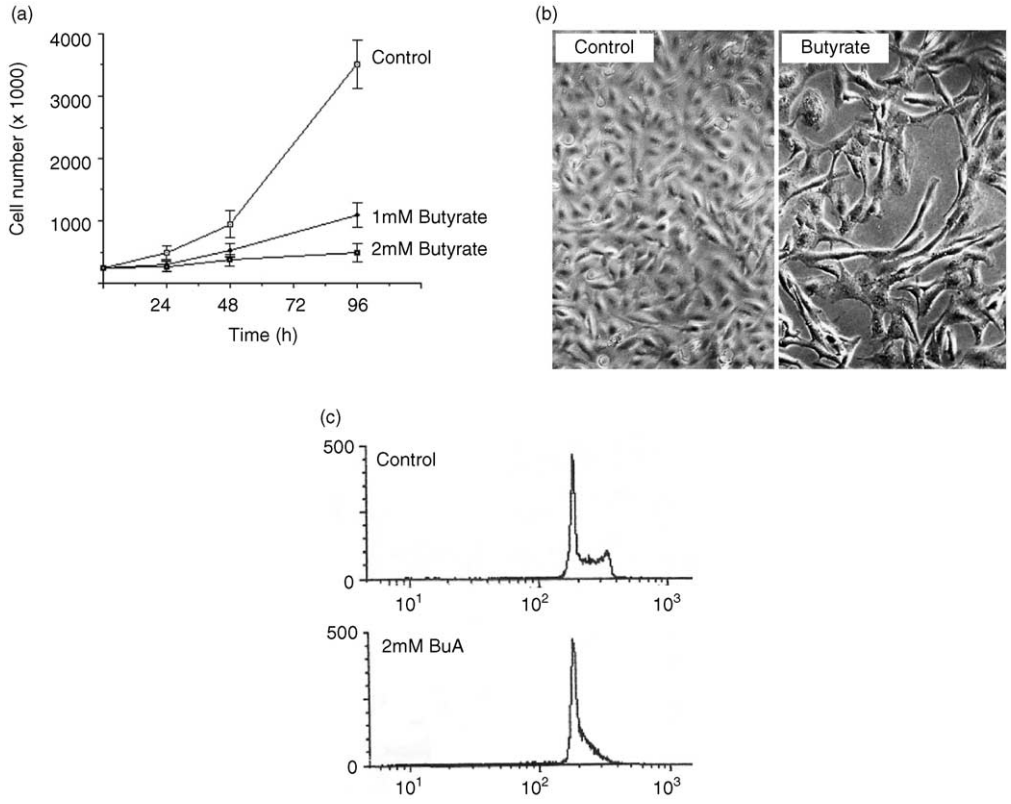

Fig. 4. Effect of butyrate on cell proliferation and morphology of rhabdomyosarcoma RH-30 cells. Panel a: RH-30 cells incubated with or without butyrate. Cells were counted at 0, 24, 48 and 96 h after the addition of the molecule. Values represent the mean±Standard Deviation (S.D.) of three experiments. Panel b: Morphology of untreated RH30 cells and cells treated with 2 mM butyrate. Panel c: RH-30 cells were grown for 48 h in the presence or absence of 2 mM butyrate (BuA). Subsequently, cells were harvested and analysed by flow cytometry as described in Ref. [29].

187, followed by an ubiquitination reaction and a proteasome-dependent cleavage [27]. Aggressive cancers appear to have a more active $p27^{Kip1}$-specific degradation system compared with neoplasias with a favourable outcome [37]. Therefore, the content of cellular $p27^{Kip1}$ is an independent prognostic factor for several cancers including tumours of colon [24], rectum [38], stomach [26], breast [23], prostate [37], liver [39] and several other tissues.

Our immunohistochemical studies (reported in Table 2) and Western blotting analyses (Fig. 3) clearly demonstrated that ARMS contained much lower levels of $p27^{Kip1}$ than the embryonal forms. Since the protein is a pivotal inhibitor of the cyclin-dependent kinases, it is well established that its presence corresponds to an elongation of the cell division cycle and to activation of the differentiation programme.

Recently, it has been demonstrated that $p27^{Kip1}$ expression is upregulated by the forkhead-transcription factor FKHR [40–42]. Interestingly, all ARMS analysed have PAX3-FKHR translocations (data not reported). It is conceivable that PAX3-FKHR and PAX7-FKHR translocations might produce chimeric proteins that hamper FKHR function by acting through a dominant-negative mechanism [40]. This, in turn, may explain (at least in part) our observations of a reduced $p27^{Kip1}$ expression in ARMS.

A second interesting finding of our studies was the higher level of $p21^{Cip1}$ mRNA observed in ERMS compared with ARMS. It is known that expression of this

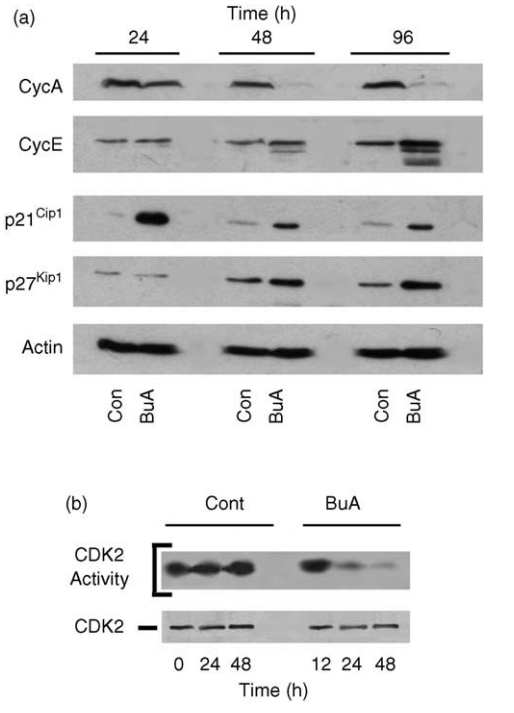

Fig. 5. Immunoblotting analysis of *Cyclin A* (CycA), *Cyclin E* (CycE), $p21^{Cip1}$ and $p27^{Kip1}$ levels in butyrate-treated RH-30 cells. Panel a: extracts from RH-30 cells incubated with or without 2 mM butyrate for different time periods (24, 48 and 96 h) were analysed for the different cell cycle proteins. Equal amounts (50 μg) of proteins were loaded in each lane. Con, untreated cells; BuA, 2 mM butyrate-treated cells. Panel b: CDK2 was immunoprecipitated from equal amounts of cellular extracts (500 μg). Identical aliquots of the immunoprecipitated materials were assayed for the kinase activity (CDK2 Activity) and for the CDK2 total content by immunoblotting (CDK2).Con, untreated cells; BuA, 2 mM butyrate-treated cells.

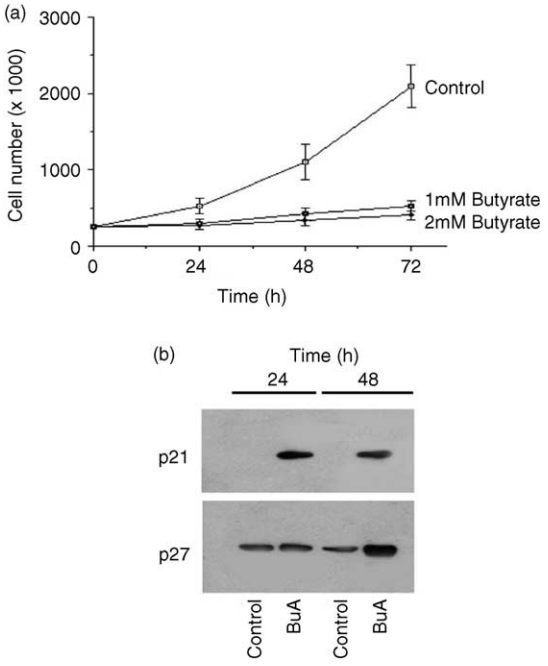

Fig. 6. Effect of butyrate on proliferation and the $p21^{Cip1}$ and $p27^{Kip1}$ levels in rhabdomyosarcoma RD cells. Panel a: RD cells were incubated with or without 2 mM butyrate. Cells were counted at 0, 24 and 48 h after the addition of butyrate. The values represent the mean±S.D. of three experiments. Panel b: Extracts from RD cells, incubated with or without 2 mM butyrate for different time periods (24 and 48 h) were analysed for the content of $p21^{Cip1}$ and $p27^{Kip1}$ levels. Equal amounts (50 μg) of proteins were loaded in each lane. Control, untreated cells; BuA, 2 mM butyrate-treated cells.

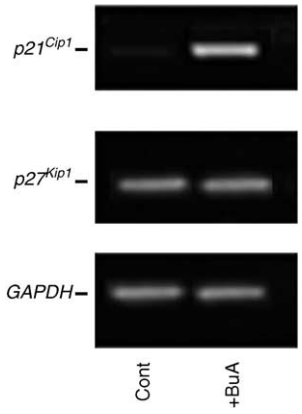

Fig. 7. Transcriptional effect of butyrate on the expression of $p21^{Cip1}$ and $p27^{Kip1}$ genes in rhabdomyosarcoma RD cells. RD cells were incubated with or without 2 mM butyrate in the presence of 36 μM cycloheximide. After 8 h, cells were collected and total RNA prepared. Then, $p21^{Cip1}$, $p27^{Kip1}$ and *G3PDH* expression was determined by RT-PCR.

inhibitor is under the control of several transcriptional factors including *TP53* and MyoD. *TP53* gene alterations have been reported in RMS [17,18] and we confirmed this observation in our specimens (manuscript in preparation). However, no correlation was observable between the *TP53* status and $p21^{Cip1}$ expression, and thus, *TP53* mutations do not appear to be responsible for the downregulation of this CDK inhibitor.

The presence of MyoD is related to the commitment of undifferentiated mesenchymal cells towards myogenesis [43]. Almost all of the samples employed in our study contained *MyoD* mRNA and protein (data not shown). However, the activity of the transcriptional factor is controlled by several mechanisms, including post-synthetic modifications (phosphorylation and acetylation) and binding with additional regulartory proteins. Thus, it is possible that the observed differences in $p21^{Cip1}$ gene expression are due to differing MyoD activities. Further studies are currently underway to clarify this point. Finally, there is currently data to suggest a correlation between the PAX-FKHR fusion protein (occurring in ARMS) and $p21^{Cip1}$ protein content and neither PAX nor FKHR appear to modulate the expression of this CDK inhibitor gene.

Recently, it has been demonstrated that a CpG site (SIE-1 element, at nucleotide (nt)-692 relative to the transcription start point) of the $p21^{Cip1}$ promoter region was completely methylated in 13 of 26 (50%) primary RMS tumours [44]. This methylation strongly correlated with a decreased $p21^{Cip1}$ expression in RMS [44]. Thus, our findings might be the mechanistic consequence of differing degrees of methylation of the $p21^{Cip1}$ promoter. This, in turn, suggests the cyclin-dependent kinase inhibitor is not a primary factor in the development/progression of RMS.

An additional intriguing result of the present study is that butyrate is able to induce cell growth arrest of two RMS cell lines and to upregulate the $p21^{Cip1}$ and $p27^{Kip1}$ protein levels. We decided to investigate the effect of the short fatty acid on the basis of a number of recent experimental and clinical observations. First of all, it has been reported that the molecule is able to upregulate $p21^{Cip1}$ content in some cell systems by acting on the expression of the gene [33]. In addition, the compound lacks any important *in vivo* toxic side-effects [45]. Finally, clinical trials are in progress in order to evaluate the usefulness of butyrate and its analogues in the treatment of several human diseases, including acute leukaemias [46], bowel inflammatory pathologies [45], β-thalassaemia [36], sickle cell anaemia [47], ornithine transcarbamylase deficiency [48], cystic fibrosis [49] and X-linked adrenoleukodystrophy [50].

The two RMS cell lines employed were selected on the basis of data from the literature which demonstrated that the $p21^{Cip1}$ gene is expressed only in RH-30 and not in RD cells [44]. The difference is due to a differing GpC methylation of the $p21^{Cip1}$ promoter [44]. The histological origin ot the cell lines (RH-30 cells from an ARMS and RD cells from an ERMS) along with the $p21^{Cip1}$ expression level are not in accordance with the findings reported in Table 1. However, it should be underlined that the RH-30 and RD cell lines were established a number of years ago and, thus, they do not necessarily represent the features of the original RMS subtypes. We selected them as experimental RMS models with the aim of verifying the possibility of increasing the CDK inhibitor levels under different conditions of $p21^{Cip1}$ promoter methylation.

As reported, butyrate induced an increase of both $p21^{Cip1}$ and $p27^{Kip1}$, with a consequent inhibition of CDK2 activity. Thus, it is highly probable that this increase in CDK inhibitor content might be responsible for the fatty acid antiproliferative effect. Furthermore, our study represents the first molecular investigation into the effects of butyrate on the cell cycle of rhabdomyosarcoma cells.

As described above, the RD cell line contains a SIE-1 methylated element which hampers $p21^{Cip1}$ expression, while the same sequence is completely unmethylated in RH-30 cells [44]. Since butyrate induced increases of the CDK inhibitors in both the cell lines (Figs. 5 and 6), it acts independently of the $p21^{Cip1}$ methylation status. This is particularly intriguing in view of the proposed use of butyrate (and its analogues) in cancer therapy [35].

In conclusion, our investigation demonstrates that two key regulators of progression through the cell cycle are regulated differently in human ERMS and ARMS. The embryonal form expressed higher levels of both $p27^{Kip1}$ and $p21^{CIP1}$. These results, along with previous data on *CDKN2A* [19] and p53 [17,18] gene inactivation and *CDK4* gene amplification [11], point to the control of cell division cycle as a key target of RMS development and/or progression. Moreover, our findings suggest that the pharmacological increase of $p27^{Kip1}$ and $p21^{CIP1}$ content, obtainable by means of butyrate or its analogues, represents a promising strategy for the development of novel treatment or this aggressive solid childhood cancer.

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