Sodium butyrate suppresses apoptosis in human Burkitt lymphomas and murine plasmacytomas bearing c-*myc* translocations

Ivan Alexandrov[a,*], Larisa Romanova[b], Frederick Mushinski[b], Richard Nordan[a]

[a] *Division of Monoclonal Antibodies, Center For Biologics Evaluation and Research, FDA, Bethesda, MD 20892, USA*
[b] *Laboratory of Genetics, National Cancer Institute, NIH, Bethesda, MD 20892, USA*

Received 6 July 1998

Abstract We report that sodium butyrate, a natural product of fiber degradation by colonic bacteria, markedly suppresses c-Myc-mediated apoptosis induced in murine plasmacytomas and human Burkitt lymphomas by growth factor deprivation, but not in cell lines devoid of c-*myc* translocations. Attenuation of cell death is associated with downregulation of the rearranged c-*myc* and activation of pRb via its dephosphorylation. We suggest that in vivo sodium butyrate may play an important role in plasmacytomagenesis by supporting the survival of cells with c-*myc* translocations, which otherwise would be eliminated by the lack of growth factors.

Key words: Plasmacytoma; Butyrate; Apoptosis; c-Myc

1. Introduction

Millimolar concentrations of sodium butyrate are produced in the large bowel of most vertebrates by colonic bacteria. It regulates the in vivo growth and maturation of colonic epithelial cells by promoting their differentiation and, eventually, their apoptosis, which is a part of their normal life cycle. It also induces growth arrest, differentiation and apoptosis in a number of colon and myeloid tumor cell lines. The latter is thought to be a molecular mechanism that could account for the correlation between a high-fiber diet and low incidence of colorectal cancer and gives a rationale for a trial of sodium butyrate as a 'differentiating' antineoplastic drug [1]. The cell cycle effects of butyrate are probably a consequence of its ability to affect the expression of various genes, including the cell cycle-related c-*fos*, cdc2, p53 and c-*myc* (both normal and rearranged) [2–5]. The ability of sodium butyrate to induce expression of the fetal hemoglobin gene has attracted attention as a potential treatment of sickle cell disease and thalassemia [6]. Association of normal fetal hemoglobin with the mutant protein increases survival of erythrocytes up to 20% compared to 4–8% in untreated cells. High (millimolar) concentrations of sodium butyrate that are suggested for treatment of colon cancer and thalassemia are rapidly metabolized in vivo and reach micromolar concentrations in plasma after 9–10 h [1].

The ability of sodium butyrate to decrease transcription of rearranged c-*myc* has attracted our attention in connection to recent studies of Myc-dependent apoptosis, which demonstrated that in the absence of growth factors the cells with deregulated c-*myc* expression are unable to shut it down and to arrest their cell cycle progression [7]. These cells undergo Myc-mediated apoptosis upon entry into the S phase of cell cycle (or later in the cell cycle). In fact, in myeloid cells [8], fibroblasts [9], EBV-transfected B lymphocytes [10], and Burkitt lymphoma cells [11] that constitutively express c-Myc, the apoptosis triggered by growth factor withdrawal is critically dependent on the presence of this protein. In these cells, apoptosis can be abrogated by downregulation of c-Myc with antisense oligonucleotides or inactivation of ectopically expressed chimeric c-Myc protein. c-Myc-mediated apoptosis may protect an organism from expansion of naturally occurring cells with deregulated c-*myc* generated as a result of tumorigenic chromosomal translocations that place c-*myc* under the control of immunoglobulin enhancers, e.g. human Burkitt lymphomas and murine plasmacytomas [12]. We suggest that a decrease of c-*myc* expression by sodium butyrate in certain circumstances might reduce apoptosis in cells carrying c-*myc* translocations and increase their chances for survival and further malignization. To test this hypothesis we have studied the effect of sodium butyrate on apoptotic cell death in murine and human B cell tumors – plasmacytomas and Burkitt lymphomas.

2. Materials and methods

2.1. Cell culture

Interleukin-6 (IL-6)-dependent murine T1165 and T1198 plasmacytoma cell lines [13] were maintained in RPMI 1640 medium (Whittaker/MA Bioproducts), supplemented with 10% heat-inactivated fetal calf serum, 50 mM 2-mercaptoethanol, 50 mg/ml gentamicin and 100 plasmacytoma growth factor units/ml of recombinant murine IL-6. Burkitt lymphoma cell lines JD38 and Daudi (American Type Culture Collection) were maintained on the same medium, but without 2-mercaptoethanol and IL-6. IL-3-dependent murine pre-B cell line Baf3 was grown on the same medium with 5% of WEHI-3 supernatant as a source of murine IL-3. IL-2-dependent murine cell lines CTLL-2 and 279 were maintained on the same medium with 10 units/ml of recombinant IL-2. To deprive cells from growth factors, they were centrifuged at $180\times g$ and washed three times with growth factor-free or serum-free (for Burkitt lymphoma cells) medium. For studies of proliferation and apoptosis, cells were seeded at a concentration of 2×10^5/ml in the same medium.

Treatment of plasmacytoma and Burkitt lymphoma cells with the different concentrations of sodium butyrate was initiated immediately after withdrawal of growth factors. Cell proliferation in the presence of growth factors was assessed by the MTT assay 48 h after the initiation of experiment. In the absence of growth factors viability was determined 24 (for T1165) and 48 h (for T1198 and Burkitt lymphomas) after the beginning of experiment. Experiments were repeated independently at least three times; each one was run in triplicate. Average numbers and standard errors are shown on the figures. The MTT assay was performed as described in [13]. In a separate series of experiments, we determined that measurements of viability obtained with direct cell counts and MTT assay paralleled each other in the presence and absence of growth factors.

*Corresponding author. Laboratory of Cytogenetics, Mental Health Research Center, Russian Academy of Medical Sciences, Zagorodnoe Sh 2, Moscow 113152, Russia. Fax: (7) (095) 952-8940.
E-mail: ivan@nlando.msk.su

PII: S0014-5793(98)00926-0

2.2. DNA extraction and analysis

5×10^6 cells were lysed for 4 h at 37°C in 400 ml of lysis buffer (200 mM Tris-HCl, pH 7.6, 1 mM EDTA, 50 μg/ml proteinase K and 1% SDS) which was followed by 1 h treatment with 50 μg/ml RNase A. After phenol-chloroform extraction and ethanol precipitation, DNA was rehydrated in TE buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA). 10–30 μg of DNA per lane were electrophoresed in 1.2% agarose gels containing 1 μg/ml of ethidium bromide.

2.3. Western blots

Western blot analysis was performed as described [13] with modifications. The following lysis buffers were used for Western blot analysis: c-Myc detection: 50 mM Tris-HCl, pH 7.2, 0.1% SDS, 50 mM dithiothreitol, 10 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, 0.3 M NaCl; for detection of other proteins NaCl in the buffer was reduced to 0.1 M. Western blots were probed with polyclonal murine-specific antibodies against c-Myc (UBI), monoclonal anti-human c-Myc (Oncogene Science; Ab-1), monoclonal anti-p53 (Oncogene Science; Ab-3), monoclonal anti-Rb (Pharmingen) and monoclonal anti-hsp70 (StressGene). Goat anti-rabbit or anti-mouse IgGs conjugated with horseradish peroxidase (Life Technologies BRL, Inc) were used as secondary antibodies. The immunoreactive bands were visualized on X-ray film using a chemiluminescent substrate (Kirkegaard and Perry Lab).

3. Results

3.1. Apoptotic cell death is induced by growth factor withdrawal in murine plasmacytoma and human Burkitt lymphoma cell lines

We have studied the process of cell death in several cell lines that carry c-*myc* chromosomal translocations, murine plasmacytomas and human Burkitt lymphomas. As we reported earlier, upon growth factor withdrawal the IL-6-dependent murine plasmacytomas T1165 and T1198 undergo apoptotic cell death with viability decreasing to 25% within 1 or 2 days, respectively. Cell death is accompanied by typical apoptotic morphology and ladder-like DNA degradation [13].

Serum removal from Burkitt lymphoma cell lines JD38 and Daudi results in apoptotic cell death within 2–3 days. Serum-starved cells displayed the typical morphological features of apoptosis as was shown by fluorescent microscopy of cells stained with acridine orange. Cells lost volume, the plasma membrane became ruffled, chromatin collapsed into crescents along the nuclear envelope, the nucleus broke up into spheres and finally cells were fragmented into apoptotic bodies (Fig. 1A). DNA isolated from serum-deprived Burkitt lymphoma cells had typical apoptotic ladders with fragments in multiples of 180–200 bp in length, whereas cells maintained with serum yielded intact high molecular weight DNA (Fig. 1B).

Thus, we have established that growth factor deprivation causes a typical apoptotic cell death in plasmacytoma and Burkitt lymphoma cell lines used in this work.

3.2. Sodium butyrate suppresses apoptosis in growth factor-starved plasmacytoma and Burkitt lymphoma cells

Sodium butyrate was proposed for treatment of several disorders by oral administration at millimolar concentrations that are naturally present in the gut. However, sodium butyrate is metabolized very quickly and reaches only micromolar concentrations in plasma a few hours after administration [1].

We have assessed the effect of physiological concentrations of sodium butyrate on cell proliferation and apoptotic death induced in mouse plasmacytoma and human Burkitt lymphoma cells by deprivation of IL-6 and serum, respectively. The highest non-toxic concentrations (250 μM for T1165 and T1198 cells; 2 mM for JD38 and Daudi cells) were used in most experiments. They were determined for each cell line by monitoring a number of dead cells in the presence of the growth factor over a period of 3 days. In the presence of growth factors sodium butyrate inhibited proliferation of all cell lines in a concentration-dependent manner (Fig. 2). In the absence of growth factors butyrate added for the whole time of growth factor withdrawal significantly suppressed apoptosis, leading to an almost complete survival of cells at the times when 50–70% of the control cells were dead (Fig. 3A). Attenuation of the apoptotic cell death was also concentration-dependent.

Prolonged treatment with sodium butyrate suppresses the apoptotic cell death induced in T1165 by withdrawal of IL-6

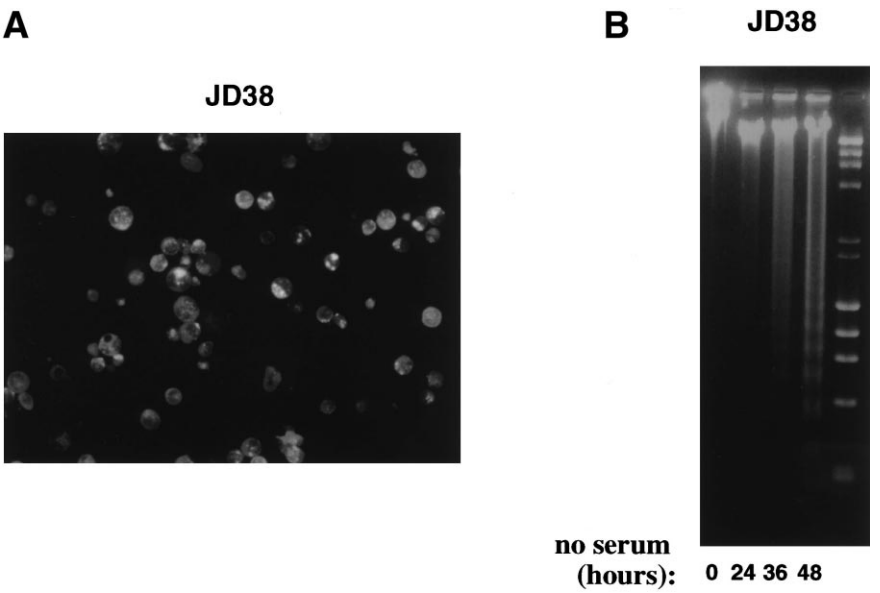

Fig. 1. Apoptotic cell death in Burkitt lymphoma JD38 cells induced by deprivation of serum for 62 h. A: Fluorescent microscopy of cells stained with acridine orange. B: DNA degradation patterns induced in JD38 cells by incubation without serum for the indicated periods of time.

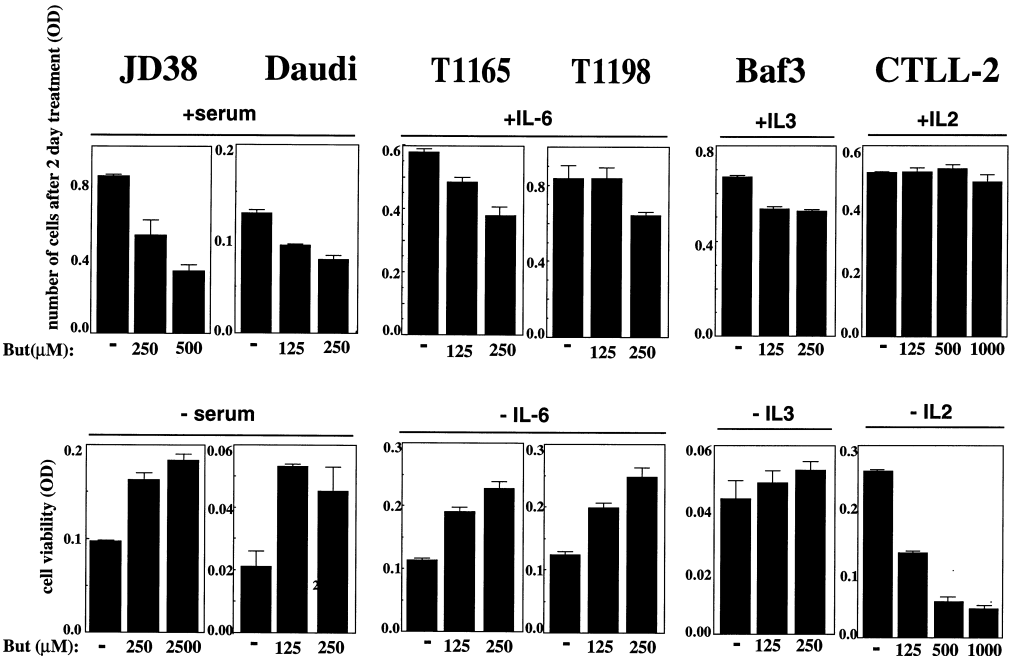

Fig. 2. Effect of non-toxic concentrations of sodium butyrate (A) on cell proliferation (in the presence of growth factors) and (B) on cell death (in the absence of growth factors). Cell viability was determined by the MTT assay at the following times after growth factor withdrawal in the presence or absence of butyrate: 24 h for T1165, Baf3 and CTLL-2, and 48 h for JD38, Daudi and T1198. At the time of measurement cell cultures were also evaluated for the presence of apoptotic cells using acridine orange staining and trypan blue exclusion methods (data not shown). Roughly two thirds of the cells were dead in growth factor-deprived samples not treated with butyrate, and no dead cells were present in samples supplied with growth factors both treated and not treated with butyrate.

(Fig. 3A). Suppression of apoptosis in these cells induced by addition of the drug for 9, 17 and 24 h, followed by wash out, was proportional to the time of treatment (Fig. 3B). Addition of sodium butyrate at 18 or 21 h after IL-6 withdrawal had no effect (not shown). We conclude that a prolonged (more than 10 h) treatment is necessary for apoptosis suppression. Tumor cell lines that do not have rearranged c-*myc* gene and exhibit Myc-independent apoptosis were used as control. The IL-3-dependent murine pre-B cell line Baf3 rapidly downregulates c-Myc after growth factor withdrawal and 1–2 days later undergoes apoptosis which therefore is c-Myc-independent [14]. The IL-2-dependent human T-cell lines CTLL-2 and 279 have a very low expression of c-*myc* (see below). They undergo apoptosis within 2 days after growth factor deprivation. In these cell lines sodium butyrate did not cause any significant attenuation of apoptosis (Fig. 2).

We conclude that sodium butyrate suppresses apoptosis in the cell lines with c-*myc* expression deregulated by chromosomal translocation, but not in the cell lines with normal c-*myc*.

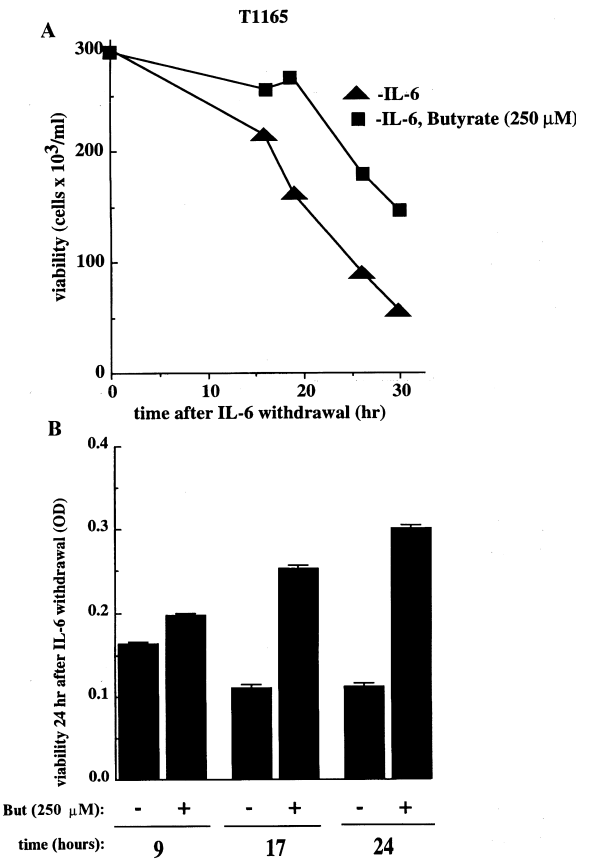

Fig. 3. A: Viability of IL-6-deprived T1165 cells with and without treatment by 250 μM sodium butyrate. Butyrate was added immediately after IL-6 withdrawal for the whole time of experiment. Live and dead cells were counted at different time points using trypan blue exclusion. B: IL-6-deprived T1165 cells were treated with 250 μM of sodium butyrate for the indicated periods of time. Then butyrate was washed out and cells were further incubated with IL-6-free medium up to 24 h. Then cell viability was determined using the MTT assay. The cell viability in the control sample, which was re-supplied with IL-6 at 12 h after IL-6 withdrawal so that the cells neither died nor proliferated extensively, was considered as 100%.

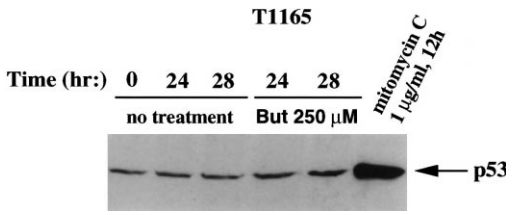

Fig. 4. The effect of sodium butyrate treatment and/or IL-6 withdrawal on p53 content in T1165 cells. The cells were incubated without IL-6 for various periods of time with or without of 250 μM of sodium butyrate. p53 content was analyzed by Western blot using monoclonal anti-p53 antibodies (Oncogene Science; Ab-3) and Hsp70 monoclonal antibodies (StressGene), as a control, on lysates prepared from the live cells purified on a Ficoll gradient. Cells maintained on IL-6 and cells stimulated with 10 mg/ml mitomycin C, which is known to induce an accumulation of p53, were included as controls.

3.3. *Apoptosis suppression by sodium butyrate is associated with downregulation of rearranged c-myc and activation of pRb but there is no change in p53*

The products of c-*myc* oncogene and of Rb and p53 tumor suppressor genes are known to be the important regulators of cell proliferation and apoptosis. We have checked if changes in Rb and p53 proteins accompany apoptosis suppression by butyrate in plasmacytoma and Burkitt lymphoma cells.

We found very little expression of p53 in T1165 plasmacytoma cells, and the level of its expression was not modulated by sodium butyrate at concentrations that were effective in cell death suppression and c-Myc modulation (Fig. 4). However, mitomycin C treatment which brings up a p53-mediated cell cycle arrest (not shown) significantly elevated p53 level in this cell line.

In Burkitt lymphoma JD38 the level of c-Myc protein first decreases upon serum deprivation and then comes up at 24–28 h, coinciding with the onset of apoptosis. The second peak of c-Myc expression was almost completely abrogated by sodium butyrate (Fig. 5). In IL-6-deprived T1165 cells the level of c-Myc did not change appreciably for at least 18 h (Fig. 5 and not shown). Treatment with 250 μM sodium butyrate for the entire period of IL-6 withdrawal decreased the level of c-Myc protein 2–3 times by 16 h (Fig. 5). Exposure to sodium butyrate for a shorter period of time had no significant effect on c-Myc expression. Thus, in both Burkitt lymphoma and plasmacytoma cells the death suppression is accompanied by a decrease of c-Myc expression and there is a temporal correlation between these two effects.

The two cell lines that exhibited Myc-independent apoptosis were also examined for c-Myc content (not shown). In Baf3, as reported previously [15], c-Myc dropped dramatically after IL-3 withdrawal and remained unchanged until the onset of cell death. IL-2-dependent CTLL-2 cells had very low level of c-Myc, both in the presence and in the absence of the growth factor, and it was not changed significantly by butyrate treatment during the time preceding the onset of apoptosis.

The pRb tumor suppressor protein is regulated by its phosphorylation state [16]. Proteins of the pRb family have been shown to be involved in the regulation of c-Myc expression [17] and function [18,19] and in the control of apoptosis [20,21]. Their phosphorylation status can be affected by sodium butyrate [22,23]. We studied the regulation of pRb by sodium butyrate using a mobility shift assay [22]. pRb nor-

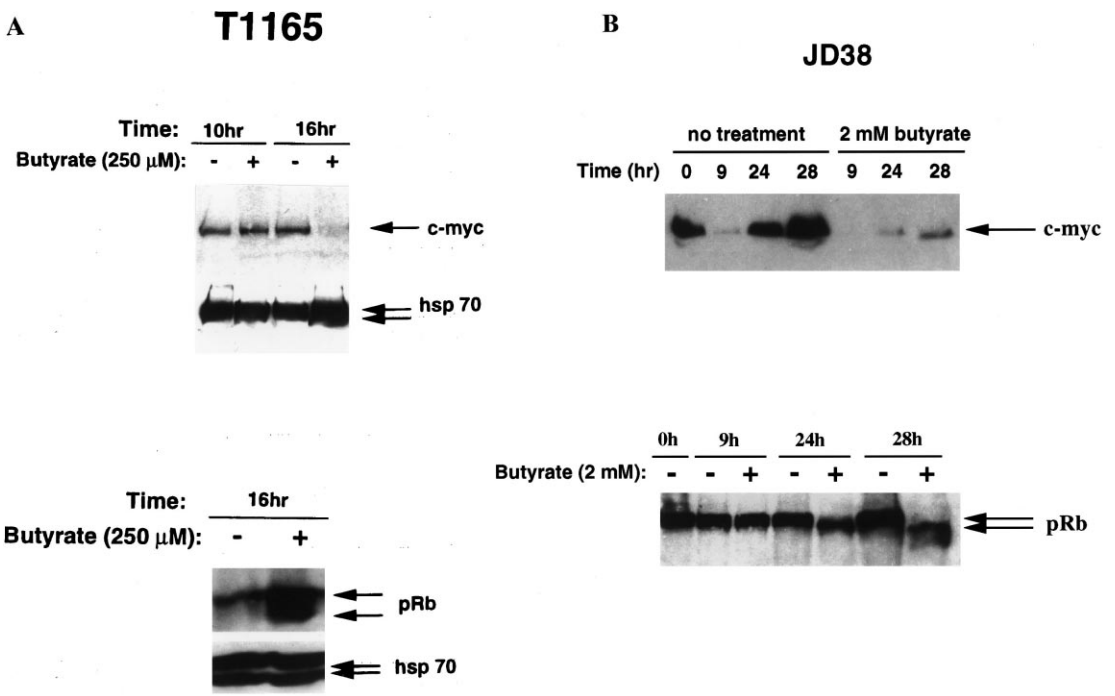

Fig. 5. The effects of sodium butyrate on c-Myc and pRb proteins in growth factor-withdrawn T1165 (A) and JD38 (B) cells. Cells were incubated in IL-6-free or serum-free medium, respectively, for the indicated periods of time, in the presence or absence of sodium butyrate. pRb, c-Myc and Hsp70 proteins were analyzed by Western blots using antibodies developed against murine pRb (Pharmingen), human c-Myc (Oncogene Science, Ab-1), murine c-Myc (UBI) and Hsp70 (StressGene).

mally appears on Western blots as a series of bands representing different phosphorylated forms. Slower moving forms are hyperphosphorylated and inactive; faster moving forms are hypophosphorylated and active in E2F binding and blocking the cell cycle (reviewed in [16]). In the case of JD38 and T1165 cells we found dramatic changes in phosphorylation state of Rb upon sodium butyrate treatment (Fig. 5). In both cases pRb was converted to high-mobility hypophosphorylated active forms. The conversion was temporally correlated with c-Myc downregulation. Also, an increase of an overall level of pRb was notable in butyrate-treated T1165 cells.

4. Discussion

Withdrawal of growth factors induces different changes in cell biology, dependent on cell type. Some cell lines downregulate their immediate early genes (including c-*myc*), develop G1/G0 arrest and undergo massive apoptosis in a short time. Others can persist in this state dying slowly over a long period of time. However, the massive apoptotic cell death can be stimulated in these cells, if c-Myc protein is ectopically expressed or deregulated in the process of oncogenic transformation. Such 'Myc-mediated' apoptosis can be induced in T-cell hybridomas [24], c-*myc*-transfected fibroblasts [15,25,26], EBV-immortalized B cells that have deregulated c-*myc* [10], and B-cell tumors with c-*myc* deregulated by chromosomal translocation [11]. In this study we found that sodium butyrate treatment, which promotes apoptosis in many previously studied systems (reviewed in [1]), has very different effects on Myc-dependent versus Myc-independent cell death. The latter is not significantly affected (Baf3 cells) or enhanced (CTLL-2) by sodium butyrate while the former is dramatically suppressed. Death suppression is accompanied by c-Myc downregulation and activation of pRb, a product of tumor suppressor gene.

Interestingly, in plasmacytoma cell line T1165 we found that modulation of p53 protein level is not involved in the cell cycle block imposed by growth factor starvation [13] or in butyrate-mediated death suppression. This adds to the body of data which shows that Myc-mediated apoptosis may occur in both p53-dependent and p53-independent ways [26,27]. However, as both pRb and p53 control arrest of cell cycle, one may speculate that in our system the former mediates apoptosis triggered by Myc, which would be consistent with the evidence showing involvement of pRb in the control of cell death in other systems [20,21].

It has been shown that downregulation of rearranged c-*myc* by sodium butyrate is achieved through a block in c-*myc* transcription [2], but its effect on pRb phosphorylation has not been investigated. It is tempting to speculate that pRb may be regulated through Myc expression, which governs synthesis of cyclins [28,29] and hence the activity of cdk/cyclin kinases which phosphorylate pRb. On the other hand, the proteins of the pRb family are able to interact with c-Myc directly [18,19], which may provide another route for pRb-Myc interplay.

As plasmacytoma tumors can be induced in BALB/c mice experimentally, they have been studied in great detail (reviewed in [12]). Plasmacytomas can be induced by pristane injections (priming) that cause a chronic inflammation – oil granuloma. They arise and proliferate in this environment which provides a continuous source of IL-6, required for these tumors at early stages of their development. Later plasmacytomas lose their dependence on IL-6 and could proliferate in mice independently of its presence or absence. As it was shown recently, cells with c-*myc* translocations could be detected in normal mice [30]. However, they do not proliferate prior to pristane injection. This allows us to propose that these cells, which apparently are IL-6-dependent and have deregulated c-*myc*, would be eliminated by apoptosis unless their survival is supported by naturally present sodium butyrate. Incidentally in non-primed mice the cells with translocations are found mostly in Peyer patches in the gut [30], where sodium butyrate is present in high concentrations. Butyrate may also play a role in supporting the cells leaving the place where the translocation has occurred and increasing their chances to find an ample supply of IL-6, proliferate and eventually progress to fully malignant, growth factor-independent tumors. Given that (i) sodium butyrate suppresses apoptosis in vitro, (ii) in many cases apoptosis suppression is known to be a crucial part of tumorigenesis and (iii) butyrate is a physiological substance and a proposed drug, we feel that investigation of its potential role in tumorigenesis in vivo is a matter of high priority.

References

[1] Newmark, H.L., Lupton, J.R. and Young, C.W. (1994) Cancer Lett. 78, 1–5.
[2] Kondoh, N., Oikawa, T., Satoh, C. and Kuzumaki, N. (1991) Exp. Cell Res. 196, 146–149.
[3] Collins, J.F., Herman, P., Schuch, C. and Bagby, G.C.J. (1992) J. Clin. Invest. 89, 1523–1527.
[4] Polack, A., Feederle, R., Klobeck, G. and Hortnagel, K. (1993) EMBO J. 12, 3913–3920.
[5] Heruth, D.P., Zirnstein, G.W., Bradley, J.F. and Rothberg, P.G. (1993) J. Biol. Chem. 268, 20466–20472.
[6] Sher, G.D., Ginder, G.D., Little, J., Yang, S., Dover, G.J. and Olivieri, N.F. (1995) New Engl. J. Med. 332, 1606–1610.
[7] Packham, G. and Cleveland, J.L. (1995) Biochim. Biophys. Acta 1242, 11–28.
[8] Askew, D.S., Ashmun, R.A., Simmons, B.C. and Cleveland, J.L. (1991) Oncogene 6, 1915–1922.
[9] Evan, G.I., Wyllie, A.H., Gilbert, C.S., Littlewood, T.D., Land, H., Brooks, M., Waters, C.M., Penn, L.Z. and Hancock, D.C. (1992) Cell 69, 119–128.
[10] Cherney, B.W., Bhatia, K. and Tosato, G. (1994) Proc. Natl. Acad. Sci. USA 91, 12967–12971.
[11] Milner, A.E., Grand, R.J., Waters, C.M. and Gregory, C.D. (1993) Oncogene 8, 3385–3391.
[12] Potter, M. and Wiener, F. (1992) Carcinogenesis 13, 1681–1697.
[13] Romanova, L.Y., Alexandrov, I.A., Schwab, G., Hilbert, D.M., Mushinski, J.F. and Nordan, R.P. (1996) Biochemistry 35, 9900–9906.
[14] Rodriguez-Tarduchy, G., Collins, M. and Lopez-Rivas, A. (1990) EMBO J. 9, 2997–3002.
[15] Shibuya, H., Yoneyama, M., Ninomiya-Tsuji, J., Matsumoto, K. and Taniguchi, T. (1992) Cell 70, 57–67.
[16] Weinberg, R.A. (1995) Cell 81, 323–330.
[17] Watanabe, S., Ishida, S., Koike, K. and Arai, K. (1995) Mol. Cell. Biol. 6, 627–636.
[18] Gu, W., Bhatia, K., Magrath, I.T., Dang, C.V. and Dalla-Favera, R. (1994) Science 264, 251–254.
[19] Beijersbergen, R.L., Kerkhoven, R.M., Zhu, L., Carlee, L., Voorhoeve, P.M. and Bernards, R. (1994) Genes Dev. 8, 2680–2690.
[20] Morgenbesser, S.D., Williams, B.O., Jacks, T. and DePinho, R.A. (1994) Nature 371, 72–74.
[21] Haas-Kogan, D.A., Kogan, S.C., Levi, D., Dazin, P., T'Ang, A., Fung, Y.K. and Israel, M.A. (1995) EMBO J. 14, 461–472.
[22] Sakamoto, K., Howard, T., Ogryzko, V., Xu, N.Z., Corsico, C.C., Jones, D.H. and Howard, B. (1993) Oncogene 8, 1887–1893.

[23] Yen, A. and Varvayanis, S. (1995) J. Cell. Physiol. 163, 502–509.
[24] Shi, Y., Glynn, J.M., Guilbert, L.J., Cotter, T.G., Bissonnette, R.P. and Green, D.R. (1992) Science 257, 212–214.
[25] Harrington, E.A., Bennett, M.R., Fanidi, A. and Evan, G.I. (1994) EMBO J. 13, 3286–3295.
[26] Hermeking, H. and Eick, D. (1994) Science 265, 2091–2093.
[27] Hsu, B., Marin, M.C., el-Naggar, A.K., Stephens, L.C., Brisbay, S. and McDonnell, T.J. (1995) Oncogene 11, 175–179.
[28] Jansen-Durr, P., Meichle, A., Steiner, P., Pagano, M., Finke, K., Botz, J., Wessbecher, J., Draetta, G. and Eilers, M. (1993) Proc. Natl. Acad. Sci. USA 90, 3685–3689.
[29] Steiner, P., Philipp, A., Lukas, J., Godden-Kent, D., Pagano, M., Mittnacht, S., Bartek, J. and Eilers, M. (1995) EMBO J. 14, 4814–4826.
[30] Muller, J.R., Jones, G.M., Janz, S. and Potter, M. (1997) Blood 89, 291–296.