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Sodium Butyrate Induces NIH3T3 Cells to Senescence-like State and Enhances Promoter Activity of $p21^{WAF/CIP1}$ in p53-Independent Manner

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Sodium butyrate, a histone deacetylase inhibitor, has been shown to induce differentiation of many cancer cells and senescence-like state of human fibroblasts. Here we show that sodium butyrate also induces senescence-like state of NIH3T3 cells. The treated cells were blocked at G1 phase and featured morphologically like senescent cells with enlarged cytoplasm and multiple nuclei. The expression of $p21^{WAF/CIP1}$ (p21) increased after sodium butyrate treatment at transcriptional level. To analyze the induction of promoter activity, we isolated 4.6 kb of murine p21 promoter and inserted it upstream of a luciferase reporter gene. When this construct was transiently transfected into NIH3T3 cells, sodium butyrate enhanced the luciferase activity. p53 independency of sodium butyrate-inducible p21 promoter activity was confirmed by using the deletion mutants lacking p53 binding sites and p53 deficient cells in transfection experiments.

Sodium butyrate has been shown to influence gene expression and differentiation in many cell lines. Cells incubated in sodium butyrate-containing medium accumulate at G1 phase, exhibit differentiated features and showed selective changes in gene expression (1-4). These effects of sodium butyrate may be resulted from its function as a histone deacetylase inhibitor (5). although the effects of sodium butyrate have been shown to be reversible, Ogryzko et. al. reported recently that sodium butyrate dramatically reduce the human diploid fibroblasts (HDFs) proliferative life span (6). The treated HDFs display markers of senescence and exhibit persistent G1 block even released from sodium butyrate. They postulated that sodium butyrate hasten entry of human diploid fibroblasts into a senescence-like state. Further, they showed that sodium butyrate-treated cells, like senescent cells, exhibit Rb gene product (pRb) hypophospholilation.

It has been shown that p21 protein inhibits the ability of G1 cyclin-dependent kinase and induces pRb hypophosphorylation, which in turn results in cell cycle arrest (7). In contrast to the low level in proliferating cells, the expression of p21 is elevated in G1 arrested or differentiated cells as well as in senescent cells (8-11). p21 expression is induced in p53 dependent manner in response to DNA damage (12), but can also be induced in the absence p53 by various agents in the absence of p53 (9, 13-15).

These works prompted us to investigate the effects of sodium butyrate to murine fibroblasts and to p21 expression. We also examined the effect of p53 to sodium butyrate induced p21 promoter activity.

MATERIALS AND METHODS

Reagent. Sodium butyrate (Wako Japan, Osaka) stock was dissolved in distilled water with 2M of concentration and stored at 4° C.

Cell culture. NIH3T3 were maintained in a 37° C humidified atmosphere containing 5% CO_2 in DMEM supplemented with 10 % FCS. BALB/c 3T310(1) cells were p53-deficient mouse fibroblasts cell line, which were kindly donated from Dr. Levine AL (16)

Cell cycle analysis. NIH3T3 cells were tripsinized and fixed with 70% ethanol. They were consecutively stained with propidium iodide (Becton-Dickinson). These cells (20,000) were analysed by Becton-Dickinson FACScan, followed by Cell Quest software (Becton-Dickinson).

RNA extraction and RT-PCR. Total RNA from culture cells was isolated through AGPC methods (17). First strand cDNA was synthesized by using Superscript II Rnase (Gibco BRL) following the protocol from the manufacture. The primers used for PCR are as follows: p21sense, 5′AGATCCACAGCGATATCCAGAC3′, p21antisense, 5′ACACACAGAGAGAGGGCTAAGG3′ (605bP); G3PDHsense, 5′TGAAGGTCGGTGTGAACGGATTTGGC3′, G3PDHantisense, 5′CATGTAGGCCATGAGGTCCACCAC3′ (983bp).

Plasmids. The luciferase reporter plasmid containing 4.6 kb of mouse $p21^{WAF/CIP1}$ promoter region (Wild-type)was constructed by pGL3-basic vector and the DNA, which was obtained by PCR ampli-

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fication using genomic DNA extracted from NIH3T3 cells according to published sequence (Genebank accession No. U24171). They contained the region from −4542 to +113 from TATA box. The deletion mutants for p53 binding sites were generated by ligating internal deleted fragments with other parts of the wild type plasmid at stuI and AatII restriction sites for mutant 1 or stuI and NheI restriction site for mutant 2. Double mutant was constructed from the deleted fragment used for mutant 1 and the other parts of mutant 2. The internal deleted fragment for mutant 1 were obtained by PCR using sense primer 5′AAGGCCTGGGTCTGTTCTGGAATTGAAGAGG3′ and anti-sense primer 5′GAGGCCTGTCTAGGTCAGAGGATCTCTAGACATCG3′, while that for mutant 2 was obtained by PCR using sense primer 5′GAGCTCAAGGCTAGCCTGAGCTATCTGAGATCAC3′ and antisense primer 5′TCTGCTGGCAAATGGGACGTCCAATTATCGGGGTC3′.

Transfection and reporter gene assay. Cells were plated into 12-well plates at density of 100,000 cells/well and transfected with 0.5μg/well of plasmid reporter construct DNA by a standard calcium phosphate procedure. After 2 days of transfection, cells were lysed, and luciferase activity in the lysates was assayed by luminometer using Luciferase assay kit (Promega). Control transfection with the parental vector were also carried out for determination of basal luciferase activity. The luciferase activities were normalized based on protein concentrations.

RESULTS

1. *Sodium Butyrate Induces Morphological Senescence-like State*

NIH3T3 cells stopped growing in the medium containing 5-10mM of sodium butyrate. Flow cytometry

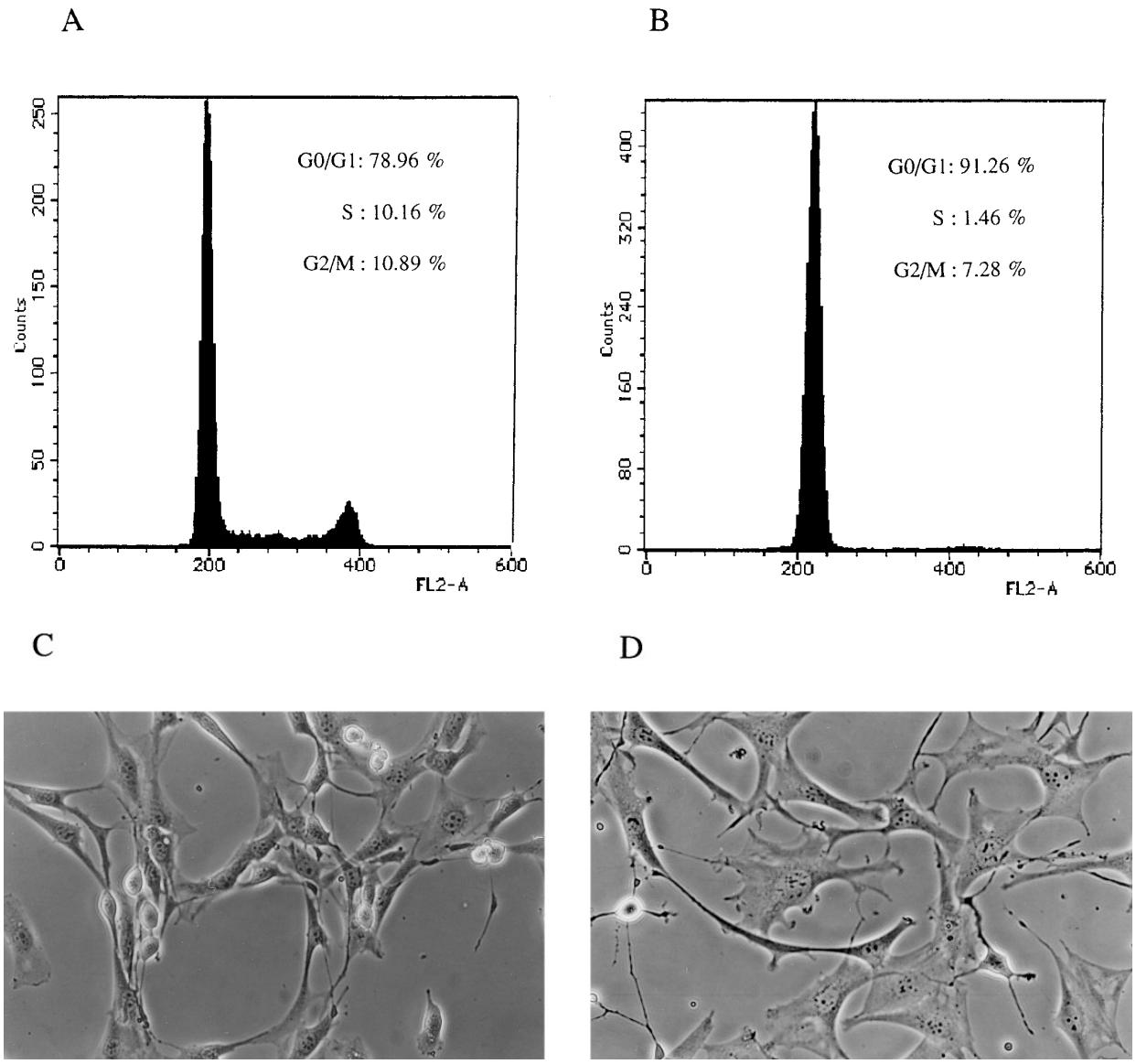

FIG. 1. A and B: Cell cycle determination by flow cytometry. Cells were stained with propidium iodide. A. untreated cells. B. Cells treated with 5 mM sodium butyrate for 24 hours. C and D: Morphologic changes. Phase contrast microscopy were taken after 2 days of 5 mM sodium butyrate treatment. C. untreated (original magnification ×100) D. Treated (original magnification ×100).

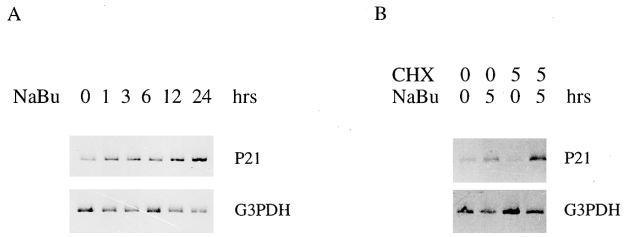

FIG. 2. Expression of p21 mRNA (RT-PCR). NIH3T3 cells were treated with 10mM sodium butyrate. A: At the indicated time, cells were collected and used to prepare RNA for RT-PCR. B: Cells were collected after 5 hours of sodium butyrate (10mM) with or without cyclohexamide (1μg/ml) treatment. RT-PCR was performed with the primers described in MATERIALS AND METHODS. G3PDH was used as control.

analysis showed that they were blocked at the G0/G1 after 24 hours of sodium butyrate treatment (Fig.1). The morphological changes of sodium butyrate treated cells were also observed. The treated cells lost the features of normal fibroblasts, such as single nucleus and scattering mitotic cells, and looked like senescent cells. They were in star-like shape with flattened cytoplasm and multinucleated (Fig.1). Almost no mitotic cells can be observed in the treated culture.

2. Sodium Butyrate Induces p21 mRNA Expression and p21 Promoter Activity

The cellular senescence has been shown to be related to p21 overproduction. We checked the induction of p21mRNA by sodium butyrate by PCR method with G3PDH as control. As shown in Fig. 2A, 10 mM sodium butyrate increased the expression of p21 mRNA. This effect was also observed in the presence of protein synthesis inhibitor cyclohexamide (Fig.2B). This result indicates that sodium butyrate induces p21 expression via transcriptional regulation. To determine whether sodium butyrate increase the transcription of p21 promoter, the construct containing 4.6 kb murine p21 promoter (wild type) in front of a luciferase reporter gene was transfected into NIH3T3 cells with or without treatment of sodium butyrate. The luciferase activity was enhanced more than 3.5 folds by the treatment of sodium butyrate (Fig.3A). The luciferase activity was started to be enhanced at 3 hours after treatment of sodium butyrate and the enhancement was gradually increased during 24 hours (data not shown).

3. Sodium Butyrate Induces p21 Promoter Activity in p53 Independent Manner

The p53 binding sites are situated near nt -2800 and nt -1900 reletive to TATA box of p21 promoter. We constructed three p53 deletion mutants for luciferase assay, which lacked either nt -2800 site (mutant 1) or nt -1900 site (mutant 2), or both sites (double mutant), and transfected them into NIH3T3 cells. As showed in Fig. 3A, the basal transcription activity of mutant 1 or mutant 2 decreased to less than 10% of that of wild type construct, while the induced luciferase activity by sodium butyrate in any of these deletion constructs was obvious and higher than that of wild type (Fig.3A). p53-deficient mouse fibroblasts cell line (BALB/c3T310(1)) was used for confirming the p53-independency of butyrate effect on p21 promoter activity. When these cells were tranfected with wild type promoter-containing construct, sodium butyrate inducible luciferase activity was observed (Fig.3B) in a time and dose dependent way (data not shown).

DISCUSSION

We showed in this report that sodium butyrate induces cellular senescence-like phenotype in NIH3T3 cells and enhances p21 promoter activity in this cell line. Although effective concentration (0.5mM) of sodium butyrate to human fibroblasts (WI-38) was found to be unaffected to the proliferation of NIH3T3 cells as reported by Ogryzko (6), we found that 5mM of sodium butyrate really blocked the growth of NIH3T3 cells. The different sensitivities to butyrate seems to be cell line-dependent. Although human diploid fibroblasts display markers of senescence and exhibit persistent G1 block even release from sodium butyrate (6), the growth arrest of NIH3T3 cells induced by short time treatment of butyrate is reversible (data not shown). Considering the facts that immortalization is a common property of murine fibroblasts and our observation that the morphologic recovery after butyrate releasing is not uniform in NIH3T3 cells (data not shown) and a small population of cells were blocked at G2/M phase (Fig.1B), we prefer to hypothesize that the reversible phenomenon is the result of living competition of cultured cells rather than exclude the possibility that butyrate can induce terminal differentiation. It means that the reproliferating cells after butyrate releasing may be come from quite rare cells which was blocked at G2/M phase during treatment or became immortelized after releasing butyrate, and their proliferation masked the presence of differentiated or senescence-like cells. To clarify this hypothesis, further experiments are undergoing. We used NIH3T3 cells to study p21 promoter activity because of the difficulty of WI-38 cells to be used for DNA transfection.

The expression of p21 is induced in a p53-dependent manner in response to DNA damage (10), but can also be induced in the absence of p53 by vitamin D3, growth factors, phobol ester and so on (9, 13-15). Two p53 binding sites are situated in upstream region of p21 promote of human, rat and mouse (18). Our work presented here indicates further that although the basic activity of p21 promoter is quite dependent on p53 transactivation, the sodium butyrate inducible activity

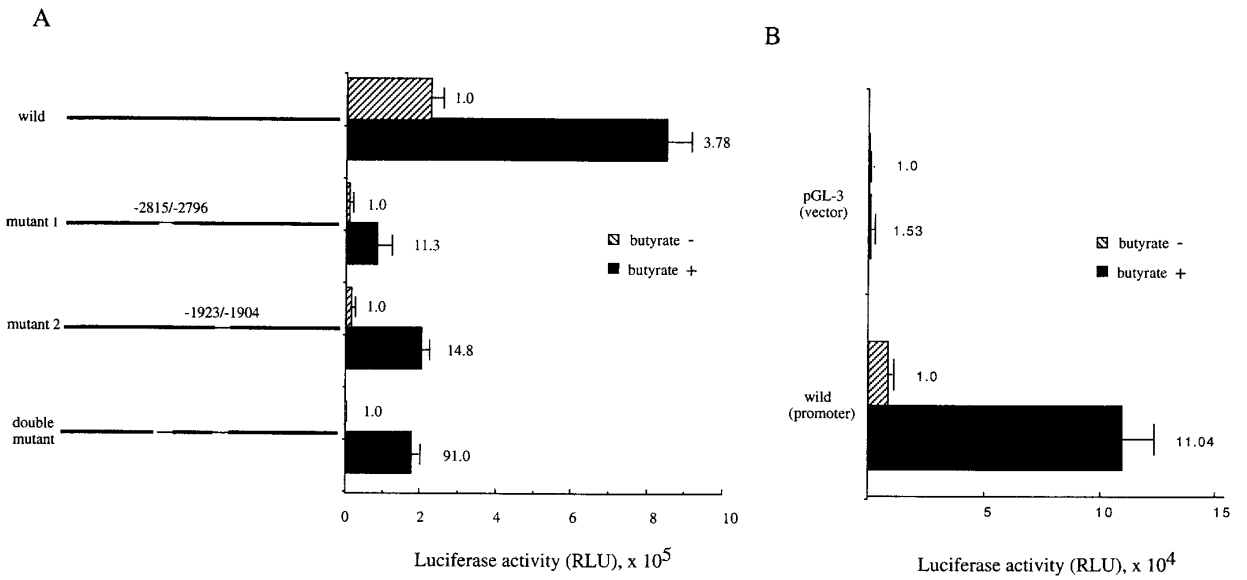

FIG. 3. p21 promoter activities. A: p53 binding site-deleted promoter/reporter constructs were transiently transfected into NIH3T3 cells with or without 10mM sodium butyrate treatment. Luciferase activity was assayed at 48 hours after transfection (12 hours after sodium butyrate treatment). B: wild type promoter/reporter construct and promoter(−) vector were transiently transfected into BALB/c3T310(1) cells with or without 10mM sodium butyrate treatment. Luciferase activity was assayed as A. These results are representative of 3 independent experiments. Data are the mean +/− SEM of two samples.

of the promoter is clearly independent on p53. This excludes one of the possible mechanisms of butyrate induced up-regulation of p21 expression, which comes from the previous finding that p53 function through associating with sequence specific DNA element by one domain and basic transcription protein, such as TBP/TAFs by another domain and then effect transcription initiation (19-20).

The exact mechanism of the regulation of p21 promoter activity by sodium butyrate remains unclear. The work for illustrating how this histone deacetylase inhibitor selectively activate p21 gene may get hint for understanding p21 gene regulation further.

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