The expression of glucose regulated protein-94 in colorectal carcinoma cells treated by sodium butyrate

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ABSTRACT

The expression of glucose regulated protein 94 (GRP94) during the treatment of human colorectal carcinoma cell line-Clone A cells with sodium butyrate was studied. Sodium butyrate (SB) can cause functional and morphological effects on Clone A cells including growth arrest at G_0/G_1 stage and cell differentiation as observed by morphological changes, MTT and flow cytometry assays, as well as reduced Grp94 gene expression as shown by Northern blot and Western blot assays. The possible mechanism of the correlation between Grp94 gene expression and tumor growth inhibition and cell differentiation is briefly discussed.

Key words: *HSPs, Grp94, sodium butyrate, colorectal carcinoma cells.*

INTRODUCTION

The stress proteins are the products of a very highly conserved genetic system in the cells. In mammalian cells, this system is divided into two predominant categories which appear to be structurally and functionally related: the heat shock proteins (HSPs) and the glucose-regulated proteins (GRPs). These protein sets, however, are separable basing on their differential sensitivity to different kinds of stresses[1],[2].

Heat shock proteins (HSPs) is a group of highly conserved proteins synthesized after heat induction. The most prominent HSPs of mammalian cells have molecular weights of approximately 90,000 (HSP90), and 70,000 (HSP70)[1],[3]. Many studies have suggested a possible correlation between the expression of HSPs and the growth and differentiation

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of tumor cells[4-9]. Recent evidence revealed that considerable expression of HSPs is found in non-heat-shocked cells, showing that HSPs may be induced by other stresses and participated in broader body defense and even in normal growth and development[10]. GRPs was also induced by various stresses to function as molecular chaperones[11],[12]. Enhanced expression of GRPs during the growth of cancer cells imply its close relationship with the cell growth[13]. However, few reports have studied the expression of *Grps* genes especially during the course of tumor growth and differentiation in comparison with those of HSPs. GRP94, an ER molecular chaperone sharing high identity with HSP90, belongs to Hsp90 gene family and participates in many important biological process as molecular chaperone[14].

Sodium butyrate (SB) has been shown to inhibit the growth and induce differentiation and apoptosis of colorectal carcinoma cells[15]. However, the molecular mechanisms that underlie butyrate-mediated growth arrest have not been fully elucidated. Therefore, we think it is worthy to investigate the *Grp94* gene expression and its possible mechanism during the treatment with sodium butyrate on colorectal carcinoma Clone A cells.

MATERIALS AND METHODS

Cell culture and cell treatment

The human colonic carcinoma Clone A cell line was a generous gift of Dana-farber Cancer Institute, Harvard Medical School, USA. Clone A cells were maintained in Dulbecco's Modified Eagle's medium supplemented with 10 % calf serum and cultured at 37ºC in humidified atmosphere containing 5 % CO_2. Clone A cells at exponential growth stage were subcultured and randomly allocated into experimental group and control group. The experimental group was added with SB dissolved in distilled water to a final concentration of 2 m M and 4 m M for 2 d and 4 d respectively, and the control group was added with the same amount of distilled water.

Observation on morphological changes of Clone A cells using LM, SEM and TEM

Clone A cells were treated with SB for 4 d and the morphological changes were observed with conventional Phase-Contrast Microscopy, Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM).

MTT assay

The effect of SB on Clone A cells were measured by MTT assay. Briefly, 5×10^4/ml cells were inoculated onto a 96-well plates in 100 μl regular medium per well and after 24 h culture, the cells of each well were exposed to SB to a final concentration of 2 m M and 4 m M for 2 d and 4 d respectively. At the end of the exposure, 10 μl (5 mg/ml) MTT in PBS was then added. After inoculation for 4 h at 37ºC, the supernatant was removed and precipitated formazan crystals were dissolved in 100 μl DMSO and the plates were shaken for 2 min. Ten min later, the cells survival rate was determined by measuring the value of OD540 with an ELISA reader (Bio-TEK Instruments Inc).

Flow-cytometric analysis

5 ml exponentially grown Clone A cells (5×10^5/ml) were plated in 25 cm² plastic flasks and

randomly divided into experimental group and control group with 3 parallel samples in each group. After 24 h, cells were treated with 2 m M SB for 2 d or 4 d. Cells were trypsinized, washed once with PBS and fixed in 1 ml ice-cold 70 % ethanol for 1 h at 4 °C. Fixed cells were pelleted, resuspended in 0.5 ml PBS containing 200 ng/ml RNase A at 37°C for 30 min and stained with propidium iodide (PI, Sigma). FACS flow cytometer fluorescence intensity was measured by FACScan flow-cytometer and the DNA content in cells was analyzed by Modfit software.

Northern blot analysis

Total RNA was prepared using TRIZOL RNA extraction kit (Gibco BRL product) from cultured Clone A cells. From each sample, 30 μg RNA was electrophoresed through a 1.5 % formaldehyde-agarose gel, then transferred to a nylon filter. The membranes were hybridized with Grp94 cDNA inserts labeled with ^{32}P-dCTP by the random primer method at 68 °C for 14-16 h. The blots were then washed at high stringency and exposed to X-ray film at -80 °C using an intensifying screen.

Specificity of anti-GRP94 antibody and Western blot assay

Rat monoclonal antibody specific for **GRP**94 (Affinity Bioreagents Inc.) was used at a dilution of 1:2000 in Western blot assay. This **GRP**94 antibody is of IgG 2 α class and was prepared by immunizing rats with purified immunogen from chicken oviducts. We confirmed the specificity of this antibody in Clone A cells using Western blot analysis. Proteins from Clone A cells were separated on a 7.5 % **SDS** polyacrylamide gel and transferred to nitrocellulose. The membranes were exposed to a rat monoclonal antibody against **GRP**94 or without **GRP**94 antibody. In addition, since GRP94 shares considerable homology to Hsp90, we checked for cross-reactivity of the GRP94 antibody with purified human liver **HSP**90 protein and the result was negative.

Western blotting analysis

Whole cells from treated and untreated Clone A cells were washed once in PBS and the cells were harvested from flasks by a rubber policeman. The cells were resuspended in 500 μl buffer, (2 mM EDTA, 10 mM EGTA, 20μM Tris.HCl pH 7.5, 1 m M Phenylmthylsufonyl floride), then sonicated and centrifuged at 100,000 $\times$ at 4°C for 1 h. The supernatants were harvested and assayed for protein concentration by the Folin-Lowry method. 100 μ g protein of each sample was denatured in SDS sample buffer and run in a 7.5 % polyacrylamide gel together with high molecular mass standards (Sigma). Proteins separated by SDS-PAGE were then electrophoretically blotted onto nitrocellulose membranes. The membrane was blocked by 5 % gelatin in Tris-buffered saline (TBS) for 1 h at room temperature and subsequently incubated with antibody for **GRP**94 (1:2000 dilution) over night. The membrane was washed with TBS to remove unbound antibody and incubated with second anti-rat immunoglobin conjugated with alkaline phosphatase. Bands were visualized with the use of alkaline phosphatase detection reagent (100 ml stock solution: 2.3 % Na2B4O7. 10 H$_2$O, 0.12 % MgSO$_4$.7H$_2$O, pH 9.7, 25 mg Tetrazotized O-dianisidine, 25 mg β4-naphthyl acid phosphate) (Sigma). The density of stained bands were quantified with an image analyzer (GDS 800, UVP product).

RESULTS

Effect of sodium butyrate on the morphology of Clone A cells

Clone A cells usually assumed an irregular or rhomboid shape, but they turned into long fibroblast-like appearance after treating with SB. The morphological changes of Clone A cells were visible in 2 d after treatment and become obvious and typical 4 d after

SB treatment (Fig 1A, B). By SEM, the control cells displayed a relatively smooth cell surface, while the treated cells grew more and longer filopodia on cell surface in 4 d as shown in Fig 1C, D. Under TEM, Clone A cells had large and irregular nucleus with at least two nucleoli and some with nuclear membrane invagination. After SB treatment, the nucleus became small and regular and nucleocytoplasmic ratio was decreased accordingly. TEM also showed that Clone A cells had fewer cytoplasm and relatively less amount of organelles, such as ER with vesicle dilation, Golgi apparatus and mitochondria etc., while in the SB-treated Clone A cells, there were relatively more cytoplasm and increased quantity of organelles. ER increased in amount and became more complex in structure, distributing around nucleus and extending through the cytoplasm. And also more Golgi apparatus and mitochondria could be seen in the cytoplasm (Fig 1E, F).
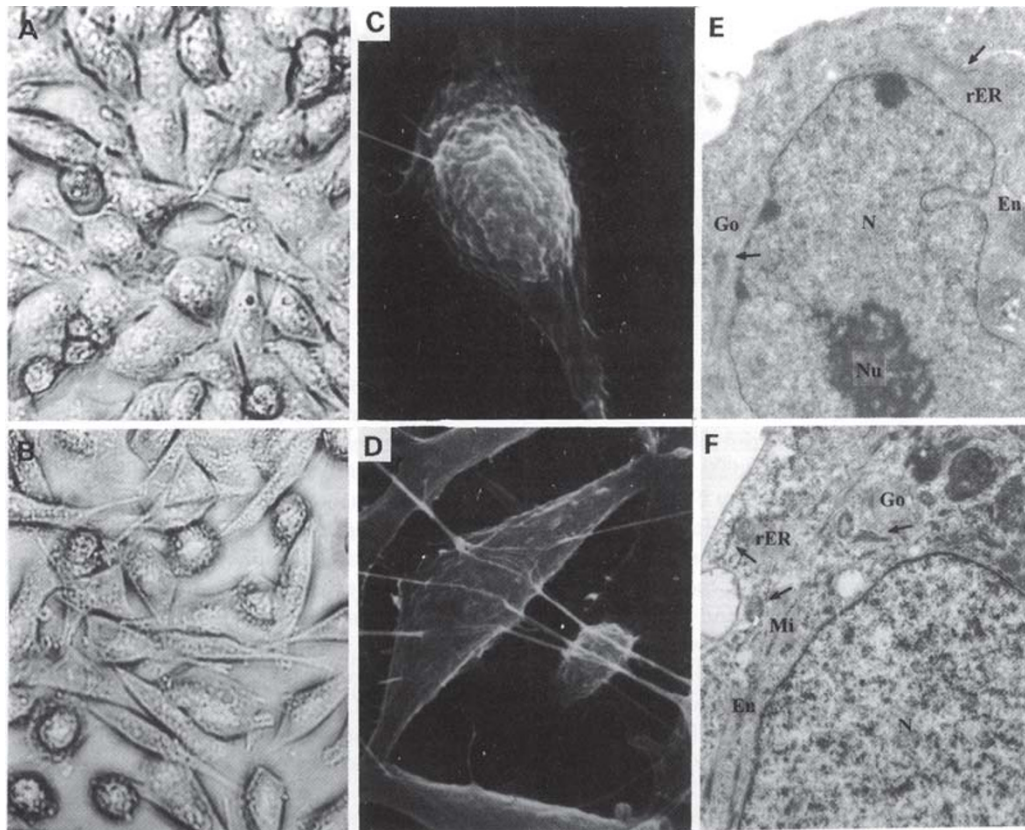

Fig 1.
Effect of sodium butyrate on the morphology of Clone A cells
A, C, E represent the morphology of control Clone A cells under the LM (200 ×), SEM (4000 ×) and TEM (10,000 ×) respectively. **B, D, F** represent morphological changes of Clone A cells treated with SB for 4 d under similar conditions.
N: nucleus; Nu: nucleoli; En: envelope; rER: rough endoplasm reticulum; Go: Golgi apparatus; Mi: mitochondria

Effect of sodium butyrate on Clone A cells survival by MTT assay

After being treated with SB in different doses (2 m M and 4 mM) for 2 d and 4 d respectively, MTT assay was used to measure the effect of SB on Clone A cells survival. It was found that growth of Clone A cells was obviously inhibited in the presence of SB, with 50 % inhibition at the concentration of 2 mM and 70 % at 4 m M, in dose-and time-dependent manner as shown in Fig 2.
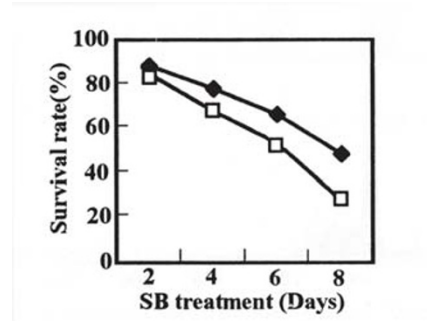

Fig 2.
Effects of sodium butyrate on Clone A survival by MTT assay. The experiment was repeated three times with similar results. Each point represents the mean of results (- ◆ - 2 m M, - □ - 4 m M).

The effect of sodium butyrate on the growth of Clone A cells and the cell cycles

In order to understand the effect of sodium butyrate on the growth of Clone A cells, flow cytometric analysis of PI stained cells was performed. Fig 3 and Tab 1 showed that the treatment of Clone A cells with a 2 mM SB for 2 d and 4 d resulted in a consistent increase in the fraction of cells in G_1 phase and decrease in S phase as compared to untreated Clone A cells.

Tab 1. DNA content analysis of Clone A treated with SB

Time (days)	SB concentration mM	Percentage of tumor cells at cell cycles			PI(%)
		G_0/G_1	S	G_2/M	
2	0	55.61	35.04	9.35	44.39
	2	61.14	30.39	8.47	38.86
4	0	55.73	36.87	7.40	44.27
	2	67.36	13.61	19.04	32.68

PI represents Proliferation Index, $PI=\dfrac{S+G_2/M}{G_0/G_1+S+G_2/M}$

Effect of sodium butyrate on Grp94 gene expression

For the study of *Grp*94 gene expression, a 270 bp product was amplified by RT-PCR with *Grp*94 specific primers and cloned into TA vector. The cDNA fragment was confirmed as Grp94 after DNA sequencing and analogue analyzing. At the same time, β-actin was used as an internal control and its 534 bp RT-PCR product was also amplified (data not shown). The effect of 2 m M SB on the expression of Grp94 was studied at the mRNA and protein levels. Northern blot analysis revealed that the amount of *Grp*94 mRNA was much higher

in untreated Clone A cells. After the treatment with SB, *Grp*94 mRNA decreased markedly with time (Fig 4). In Western blot analysis of Grp94 expression, only one band at approximately 94 KDa was identified. Clone A cells contained more *GRP*94 protein than that treated with SB (Fig 5), a result consistent with its change of *Grp*94 mRNA level. The bands were quantified by densitometry.
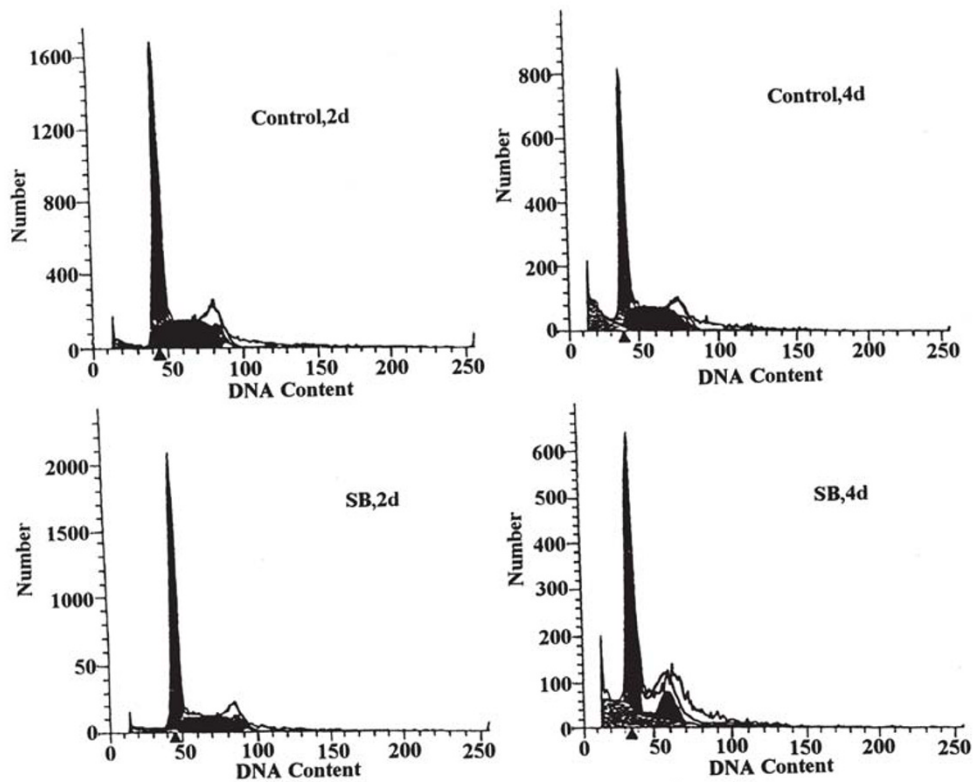

Fig 3.
Flow cytometric analysis of growth inhibition of Clone A cells in G_1 phase following exposure to 2 mM SB for 2 d and 4 d. The experiment was repeated three times with similar results.

▷ **Fig 5.**
Western blot assay to detect GRP94 protein with specific rat monoclonal antibody against GRP94 in Clone A cells after the treatment with SB.
Clone A cells were treated with SB for 2 d and for 4 d. 100 μg of extracted proteins were loaded and run in 7.5 % SDS-polyacrylamide gels and later transferred to nitrocellulose membranes, which were probed with anti-GRP94 antibody. A: Detection of specificity of GRP94 antibody by Western blots. A single band at approximately 94 KDa was identified in lane 1 and no band was found without exposing to antibody against GRP94 (lane 2). B: Western blots of Clone A cells treated with SB, showing the reduction of GRP94 protein content. C: The densitometric analysis of the data presented in B, indicating that the decrease of GRP94 protein upon SB treatment is parallel to that of Grp94 mRNA as shown in Fig 4.
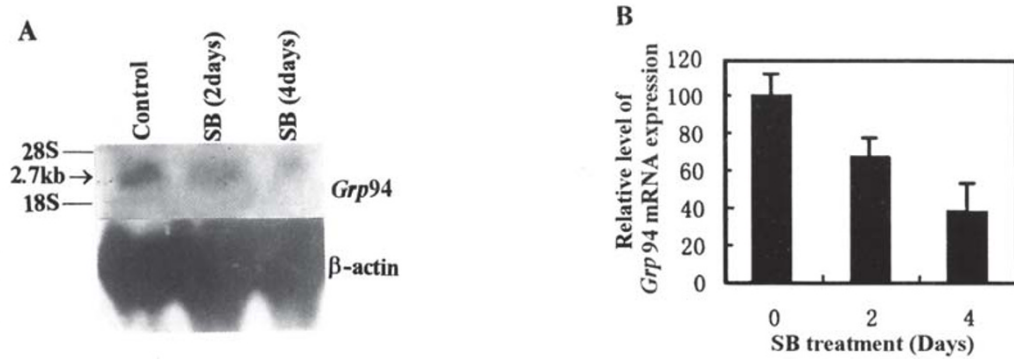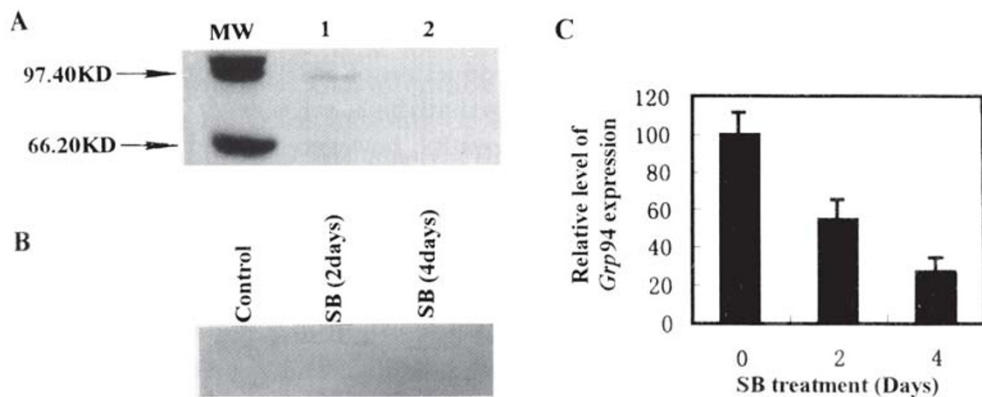
A

28S —
2.7kb →
18S —

Control   SB (2days)   SB (4days)

*Grp*94

β-actin

B

Relative level of *Grp*94 mRNA expression

120
100
80
60
40
20
0

0   2   4
SB treatment (Days)

Fig 4.

Effect of sodium butyrate on *Grp*94 expression in Clone A cells by Northern blot analysis. 30 μg total RNA were separated by 1.5 % agarose gel electrophoresis, and Northern blot was performed using *Grp*94 cDNA probe and β-actin probe labeled with ^{32}P-dCTP by the random primer method.

A: Each lane was loaded with 30 μg total RNA of Clone A cells. From left to right, lane 1 without SB treatment, lane 2, 3 treated with SB for 2 and for 4 d respectively. The size of hybridization band was approximately deduced by comparing the distance from loading well to the band of 28 S rRNA (6.3 kb) and 18 S rRNA (2.3 kb) with that from hybridization band to loading well. The positions of the 28 S and 18 S ribosomal RNAs are indicated at the margins.

B: The amount of mRNA were grossly quantified by densitometry and represented as percentage change of Grp94 expression with control cells arbitrarily taken as 100 %, mean ± SD (n ⩾ 3 in all cases).

A

97.40KD →
66.20KD →

MW   1   2

B

Control   SB (2days)   SB (4days)

C

Relative level of *Grp*94 expression

120
100
80
60
40
20
0

0   2   4
SB treatment (Days)

DISCUSSION

In the present paper, we studied the effect of sodium butyrate on poorly differentiated human colorectal carcinoma Clone A cells. The results indicated that the growth of Clone A cells is obviously inhibited and cells are arrested at G_0/G_1 phase of cell cycle after SB treatment, a fact which could be considered as an early event in cell differentiation. The morphological change of Clone A cells from irregular and rhomboid shape to a long fibroblast-like appearance was consistent with results found in the previous report[16]. The organelles, such as ER, Golgi apparatus and mitochondria increased in amount and became more complex in structure after SB treatment. These results provided evidence that SB could induce the differentiation of Clone A cells and lead to their structural alterations[17],[18].

With respect to the expression of *Grp*94 gene in Clone A cells under this condition, it was found that considerable level of *Grp*94 mRNA is present in the Clone A cells as shown by Northern blot analysis, but the amount of mRNA reduces drastically in a time-dependent manner after the treatment with SB. The change of the amount of protein encoded by *Grp*94 gene is also consistent with the change of its mRNA level as shown in Western blot analysis. In connection with the changes of *Grp*94 gene expression of colorectal carcinoma cells upon SB tratment, it is interesting to note that several studies have suggested that HSPs play some roles during cell growth and cell differentiation of tumor. The expression of the *Hsp*90 gene was enhanced in the leukemia cell lines and the acute leukemia cells as compared with normal mononuclear cells, but was markedly suppressed after tumor growth inhibition[5]. Continuous expression of HSPs proteins in tumor cells may be required to serve as molecular chaperones in regulating and stabilizing tumor growth process. Several studies also revealed that some *Hsps* gene(s) were activated by differentiation inducer, suggesting their roles during differentiation of tumor cells[8], [9]. For instance, an increase in the mRNA or protein expression of *Hsp*70 gene was shown during the differentiation of human leukemic cells to erythrocytes, monocytes or macrophages and the inhibition in the production of HSP70 protein could reduce the accumulation of hemoglobin, indicating that the latter is needed for full differentiation capability of K562 cells[8]. Other results, however, implied that some HSPs are not strictly required for differentiation in spite of their high level in differentiated tumor cells[19]. All these findings have no satisfactory explanation yet. It is not entirely clear whether agent that induce differentiation may also induces a stress response or if differentiation process per se involves the induction of HSPs.

GRPs, sharing highly in amino acid identity to HSPs, are thought to act as molecular chaperone, helping in transporting, folding and processing of their target proteins. The *Grp*78 and *Grp*94 are members of the *Hsp*70 and *Hsp*90 gene family respectively, and available evidence indicates that members of each family express analogous functions and physico-chemical characteristics. However, GRPs do differ from the HSPs proteins which are localized in cytoplasm and mitochondria, whereas, GRPs are mostly located in

endoplasmic reticulum and nuclear envelope. Furthermore, GRP94 has also been identified as a murine tumor rejection antigen, indicating that to some extent it can also reside on the cell surface[20]. It has been shown that large amount of GRPs proteins were synthesized during tumor growth, HSP90 protein, in this case, was repressed[13]. The possible explanation might be that GRPs proteins is likely to be triggered by abnormalities in protein processing or folding in the endoplasmic reticulum, which, in some cases, has been shown to be resulted from abnormal glycosylation. Although the cellular implication of the increased production of these proteins is unknown, it may be expected that each response would enhance the capacity of the pathway(s) in which these proteins function and perhaps protect the associated cellular compartments from damage via abnormal protein interactions. This could also be inferred from the findings that inhibition of GRPs expression in vitro retards cell growth and lowers its viability after ionophore exposure[21], and the preinduction of GRPs can enhance the tolerance of cells to stress stimuli[22]. In present study, the observed fact that the poorly differentiated colorectal carcinoma Clone A cells can synthesize more *Grp*94 is understandable from the above reasoning and this experimental system may be used to study the correlation between *Grp*94 gene expression and tumor growth.

The molecular mechanism involved in the change of *Grp*94 gene expression during the SB treatment of Clone A cells is unclear. But it has been clarified that, like heat shock genes, promoter region of *Grp*94 gene contains a short, highly conserved sequence, the heat shock element (HSE), implying that the heat shock factors (HSF) may also be the key transcription factors in the regulation of *Grp*94 gene expression[23]. In addition, the precise molecular mechanism that mediates SB growth inhibition is also poorly understood. But it is known that SB can cause predominantly a histone H4 hyperacetylation through inhibition of the histone deacetylase enzyme, allowing the accessibility of transcription factor to turn on specific gene(s), such as p21 gene, which is a tumor suppressor gene and also a cell-cycle suppressor[24],[25]. But how to account for the down-regulation of *Grp*94 gene expression after treatment with SB? Can alteration of certain gene(s) directly suppress *Grp*94 gene via histone hyperacetylation? Or alteration of certain molecular events that can indirectly suppress *Grp*94 gene via the process of differentiation and maturation in induced Clone A cells? All these problems need further studies.

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