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N-Acetylcysteine Increases the Biosynthesis of Recombinant EPO in Apoptotic Chinese Hamster Ovary Cells

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Sodium butyrate (NaBu) is known to enhance the rate of biosynthesis of recombinant proteins in Chinese hamster ovary cells (CHO). Here we demonstrate that supplementation with NaBu during rapid growth brings about abrupt death of the cells. The death of the cells is due to apoptosis, as assessed by intranucleosomal DNA fragmentation. The promotion of apoptotic death of the cells could be partially blocked by treatment with the well-known antioxidant, N-acetylcysteine (NAC). Strikingly, the NAC treatment enhanced the production of recombinant EPO two-fold compared with that of the culture without NAC supplementation. These results showed that NaBu treatment supplemented with NAC not only inhibits apoptosis, but also exerts a synergistic effect on the biosynthesis of recombinant EPO.

Keywords: N-acetylcysteine, apoptosis, sodium butyrate, chinese hamster ovary cell, recombinant erythropoietin

INTRODUCTION

Butyrate is a common four-carbon fatty acid that has been shown to act as a potent cell growth inhibitor and inducer of differentiation.[1,2] It is also known to enhance expression of genes from some of the mammalian promoters, including the Cytomegalovirus (CMV) promoter and the SV40 (Simian virus 40) promoter.[3–6] Several recent reports have demonstrated success in using butyrate in the mass production of recombinant proteins, such as erythropoietin (EPO),[3] tissue-type plasminogen activator (tPA),[4] interferon omega,[5] glutamyltransferase,[6] α_1-antitrypsin[7] and nitric oxide synthase,[8] in recombinant Chinese hamster ovary (CHO) cells. Butyrate shows potential for the establishment of efficient expression systems for analytical purposes, as well as for the large-scale production of recombinant proteins.

Conversely, researchers have also demonstrated that butyrate can induce apoptosis in various types of cells. These cell types include human myeloid leukemic cell lines (HL-60, U-937),[9,10] human colonic carcinoma cell lines

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(HT29, SW620),[11] Burkitts lymphoma cell lines (BL-30),[12] rat intestinal epithelium[13] and many others. The toxicity of butyrate was reduced in non-mitotic cells but in the case of rapidly proliferating cells, inhibition of DNA synthesis and partial degradation of DNA led to apoptotic death of the cells.[14,15] Since the viability of the cells is important for production of recombinant proteins, the apoptotic effect of butyrate on cells can be a severe drawback to practical applications.

In this paper we describe butyrate-induced apoptosis in CHO cells and demonstrate that the death of the cells can be inhibited by N-acetylcysteine (NAC) treatment. We show that NAC can also have a positive effect on the production of recombinant EPO.

MATERIALS AND METHODS

Chemicals and Medium

Sodium butyrate was purchased from Merck Co. N-acetylcysteine was purchased from Sigma Chemical Co. Dulbecco's modified Eagle's medium (DMEM)/F12, fetal bovine serum (FBS), trypsin-EDTA and antibiotic–antimycotic solution were purchased from Life Technology Co. The other chemicals were of the highest grade available.

Cell Line and Culture Conditions

The recombinant erythropoietin (EPO)-producing CHO cell line was kindly provided by Dr. H.J. Hong of the KRIBB (Korea Research Institute of Bioscience and Biotechnology). The cell line was constructed by introduction of genomic DNA encoding human EPO under the control of the CMV promoter. Cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum containing the antibiotic–antimycotic solution in 6-well dishes (Falcon, Becton Dickinson Co.) under an atmosphere of humidified 5% CO_2 at 37°C, as reported previously.[16]

Enumeration of Cells

At the end of the culture period, cells were detached from the dishes by treatment with 0.025% trypsin-EDTA. Detached cells were stained with trypan blue for determination of the number of viable cells.

Assessment of Apoptosis

Cells were grown to log phase in 100 mm culture dishes containing 20 ml of medium and apoptosis was induced by supplementation with 5 mM NaBu. The apoptotic nature of the cells was confirmed by agarose gel electrophoresis of nuclear DNA. Cells were harvested by centrifugation at $700 \times g$ for 5 min. Cell pellets were resuspended in 0.4 ml of lysis buffer containing 50 mM Tris, pH 8.0, 10 mM EDTA, 500 μg/ml of proteinase K and 1% SDS. After incubation for 16 h at 37°C, the solubilized cell samples were extracted with saturated phenol and saturated chloroform before the addition of 0.1 volume of 3 M sodium acetate, pH 5.5. Extracted samples were further precipitated in 2.5 volumes of cold ethanol, rinsed in 70% ethanol and dissolved in TE buffer containing 10 mM Tris, pH 7.4 and 1 mM EDTA. RNA was hydrolyzed by treatment with RNase A (20 μg/ml) at 37°C for 16 h. To evaluate DNA fragmentation, 1.5 μg of DNA samples were electrophoresed in each lane of a 1.8% agarose gel at 80 V for 3.5 h, stained with 50 μg/ml of ethidium bromide and visualized under UV.

Quantification of Fragmented DNA

The fragmented DNA was measured as described by McConkey *et al.*[17] Cells treated with NaBu were lysed in 0.33 ml of buffer containing 5 mM Tris, pH 8.0, 20 mM EDTA and 0.5%(w/v) Triton X-100. After incubating for 15 min on ice, samples were centrifuged for 20 min at $27,000 \times g$ to separate the intact chromatin (pellet) from the fragmented DNA (supernatant). Pellets were resuspended in 0.33 ml of buffer containing

10 mM Tris, pH 8.0 and 1 mM EDTA. Pellet and supernatant samples were assayed for DNA content using diphenylamine. A sample containing DNA was adjusted to a final concentration of 0.5 N perchloric acid and DNA was extracted by heating for 20 min at 70°C. The samples were mixed with 2 volumes of diphenylamine reagent containing 1.5% diphenylamine(w/v), 1.5% sulfuric acid(v/v) and 0.008% acetylaldehyde(v/v) in glacial acetic acid. After incubation for 17 hr at 30°C, the A_{600} was measured using a spectrophotometer. Fragmented DNA was quantified by measuring the ratio of the DNA content in the supernatant fraction to the total (supernatant plus pellet) DNA content.

EPO Assay

EPO concentrations in the supernatant were measured using an indirect ELISA (enzyme-linked immunosorbant assay). Anti-EPO monoclonal antibody was purified from the supernatant of a hybridoma (ATCC HB 82C9 5F3AD) culture medium using a Protein G column (Pharmacia). Recombinant EPO was purchased from Boehringer Mannheim for use as an EPO standard. Anti-mouse monoclonal antibody conjugated to horseradish peroxidase (Southern Biotechnology Associates, Inc.) was used as a secondary antibody. The A_{490} of the colored substrate (*O*-pheylenediamine) was measured using an ELISA plate reader.

RESULTS

Assessment of Apoptosis

Intranucleosomal DNA fragmentation was used to assess the induction of apoptosis caused by exposure of the cells to NaBu. Cells were initially grown in 100 mm dishes in medium lacking NaBu (initial cell density: 2×10^6 cells/dish). In the middle of the rapid growth phase (after 48 h), the cell medium was exchanged for fresh medium containing 5 mM NaBu. After 12 h of culture, cells were harvested for isolation of the intranucleosomal DNA by the procedures described previously ('assessment of apoptosis' in Materials and Methods). The results of the gel electrophoresis clearly showed the fragmented pattern of DNA that implies induction of apoptosis (Figure 1, lane 4). Cells exposed to UV-irradiation (40 J/m^2) for induction of apoptotic conditions[18] showed an identical fragmentation pattern (Figure 1, lane 5). The cells exposed to 5 mM NaBu throughout the culture period did not show the apoptotic pattern of DNA fragmentation (Figure 1, lane 3). This result indicates that NaBu promotes apoptosis in rapidly proliferating CHO cells.

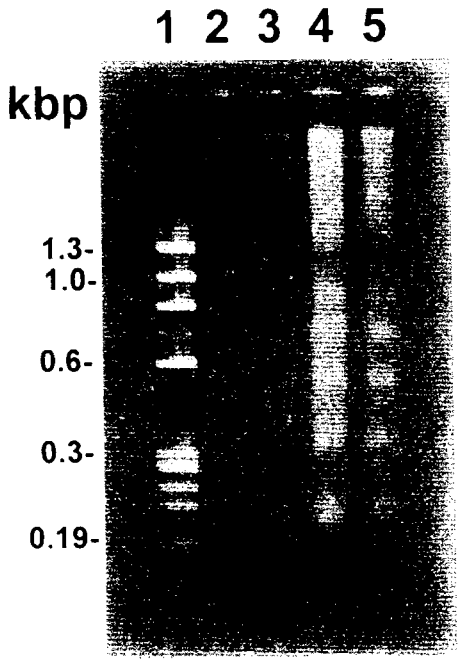

FIGURE 1 Assessment of apoptosis by agarose gel electrophoresis of DNA extracted from cells cultured under various conditions. Lane 1, size marker; lane 2, DNA from control cells; lane 3, DNA from cells exposed to NaBu from the beginning of culture; lane 4, DNA from cells exposed to NaBu during the rapidly proliferating stage; lane 5, DNA from cells irradiated with UV (40 J/m^2).

Progression of Apoptosis

Time-course studies further confirmed the promotion of apoptosis. The progression of apoptosis

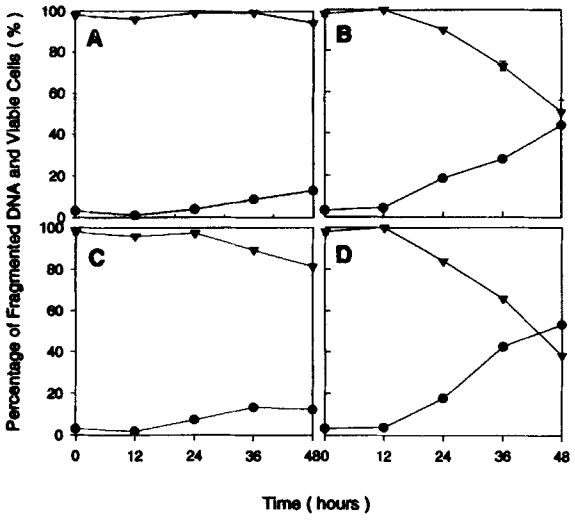

FIGURE 2 Time-course of the promotion of apoptosis by NaBu treatment. The promotion of apoptosis of cells was monitored without NaBu treatment (A), after treatment with NaBu (B), when NaBu was added at the beginning of the culture period (C), and after irradiation with UV ($40\,J/m^2$) light (D). Closed triangles (▲) represent the percentage of viable cells and closed circles (●) represent the percentage of DNA fragmentation. The points are the mean of triplicate assays and error bars represent the standard deviation. Where no error bar is present, the standard deviation was smaller than the symbol.

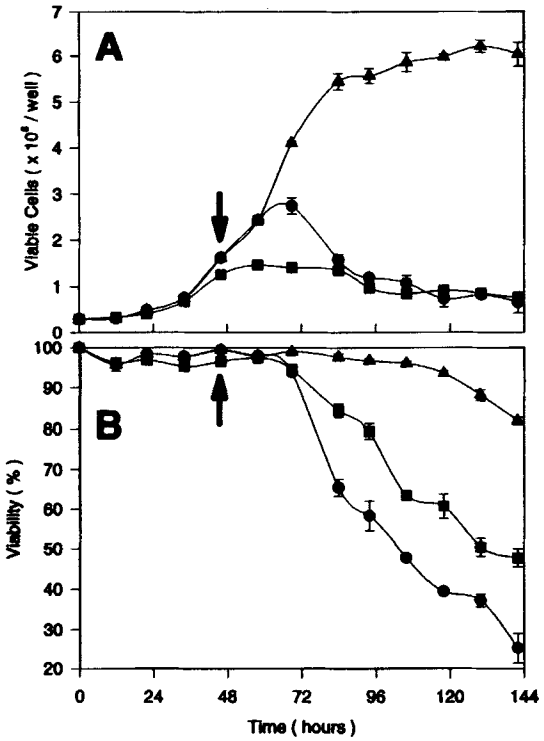

FIGURE 3 Growth and viability of cells under various conditions. Panel A shows the growth of the cells and panel B shows the viability of the cells. Closed triangles (▲) indicate control cell cultures, closed circles (●) indicate cultures supplemented with 5 mM NaBu alone, and closed squares (■) indicate cultures supplemented with both 20 mM NAC and 5 mM NaBu. The arrow indicates the time of treatment with NaBu. The points are the mean of triplicate assays and the error bars represent the standard deviation.

was monitored by assessment of two parameters: (1) viability of cells and (2) the extent of DNA fragmentation. Cells were cultured and monitored for 48 h under the conditions described above. The two parameters showed a complementary relationship. The viability declined concomitantly with an increase in fragmented DNA. Rapidly proliferating cells were more vulnerable to NaBu treatment (Figure 2B). The decline in viability and the increase in the amount of fragmented DNA was comparable to that observed for UV-irradiated cells (Figure 2D).

Effect of NAC on Apoptotic Death of Cells

Since cell death may have a considerable effect on the production of recombinant protein, we used the well-known antioxidant, NAC, to prevent apoptosis. A time-course study was performed to examine the effect of NAC on apoptosis. 0.5×10^5 cells per well were incubated in 6-well plates containing 5 ml of the medium described in 'Materials and Methods'. NAC (20 mM) was added to cells at the beginning of the culture period and exchanged for medium containing both 5 mM NaBu and 20 mM NAC after 48 h. Sampling was performed every 12 h for 6 days. In the case of cells exposed to NaBu without NAC, a sharp decline in the number of viable cells was observed, beginning 24 h after the NaBu treatment (Figure 3A). This implies that NaBu is toxic to the rapidly growing cells. In the case of cells treated with both NaBu and NAC, while growth of cells was suppressed by NAC (Figure 3A), the decline in cell viability was much slower than that

observed in cultures without NAC (Figure 3B). This demonstrates that, to some extent, NAC blocks the promotion of apoptosis by NaBu.

Effect of NAC on the Biosynthesis of Recombinant EPO

Even though cell growth in NAC-treated cultures was at a consistently low level, expression of the recombinant EPO was remarkably high compared to cultures without NAC treatment (Figure 4). Cultures treated with NaBu alone produced an approximate two-fold higher concentration of EPO compared to the control. Cultures treated with both NaBu and NAC showed an enhanced productivity that was still two-fold higher in final concentration when compared to the culture treated with NaBu alone. Culture treated with NAC alone was also effective in production of EPO (Figure 5). Cells treated with 10 and 20 mM NAC showed increased production compared to the control. These results indicate that treatment with NAC and NaBu in combination exerted a synergistic effect on the biosynthesis of recombinant EPO.

Morphological Alteration of Cells in NAC-treated Cultures

Cells were cultured for 48 h in medium with or without NAC and their morphology was examined using phase-contrast microscopy (Figure 6A and B). Cells cultured in 20 mM NAC lost their polarity and spread in an irregular manner (Figure 6B). The borders between cells were indistinguishable and large number of vacuoles formed in the cell cytoplasm. These features suggest that NAC also induces cell differentiation.

DISCUSSION

In this report, we showed that NaBu treatment of CHO cells promotes apoptosis and that cell death can be partially blocked by treatment with NAC. Moreover, the production of recombinant

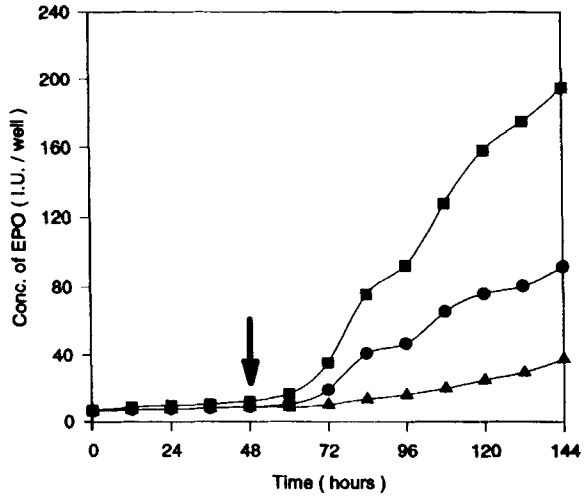

FIGURE 4 Cumulative production of EPO under various culture conditions. Closed triangles (▲) indicate control cell cultures, closed circles (●) indicate cultures supplemented with 5 mM NaBu alone, and closed squares (■) indicate cultures supplemented with both 20 mM NAC and 5 mM NaBu. The arrow indicates the time of treatment with NaBu. The points are the mean of triplicate assays.

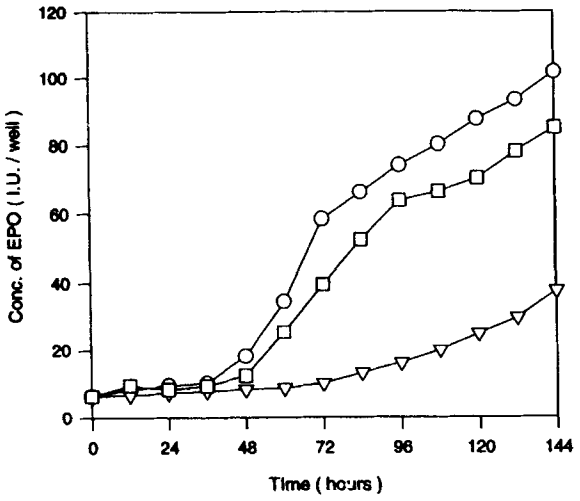

FIGURE 5 Cumulative production of EPO in cultures treated with NAC only. Open triangles (▽) indicate control cell cultures, open circles (○) indicate cultures supplemented with 10 mM NAC, and open squares (□) indicate cultures supplemented with 20 mM NAC. The points are the mean of triplicate assays.

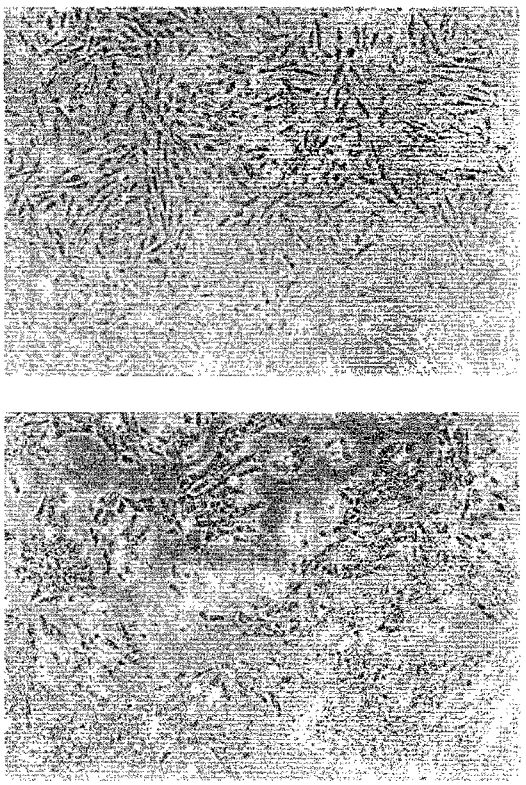

FIGURE 6 Morphology of recombinant CHO cells maintained in medium with or without NAC. Recombinant CHO cells were maintained for 48 h in 10% fetal bovine serum containing DMEM/F12 medium either without NAC (A) or supplemented with 20 mM NAC (B). Photographs were taken under a phase-contrast microscope. Magnification: 300×

EPO was improved by the NAC treatment. We observed two novel features of CHO cells and their apoptotic nature, which are noted below.

Though it promotes the expression of high levels of recombinant proteins, relatively low concentrations (5 mM) of NaBu also induce apoptosis in rapidly proliferating CHO cells. Recently, several studies have examined the production of recombinant proteins by NaBu-treated CHO cells,[3–8] but none of these discussed cell death or warned of the problems caused by NaBu. Some of these studies[3–7] used lethal amounts (above 5 mM) of NaBu in cultures to which NaBu was initially added in order to prevent cell death. Under these conditions, the arrest of cell growth could not be avoided. The mechanism by which butyrate induces apoptosis is controversial. Butyrate arrests colon cancer cells in G1,[19] inhibits histone deacetylase,[20] and down-regulates the transcription of bcl-2.[21] Moreover, butyrate was recently found to generate reactive oxygen species (ROS), which appear to trigger apoptosis.[22] Giardina *et al.* reported that butyrate induces peroxide production, which was detected using dihydrodichlorofluorescein (H_2DCF), a peroxide-sensitive probe. Although ROS may not be essential for apoptosis, there is evidence that ROS play a role in apoptosis triggered by a large number of stimuli.[23] Taking these facts into account, NAC may also block butyrate-induced apoptosis via increasing expression of glutathione peroxidase, as in the other reports.[23,25] The properties of NAC are distinct from those of other common antioxidants, while some thiol reductants (including 2-mercaptoethane sulfonic acid and thioglycolate) share similar properties with NAC.[24,28] The prevention of apoptosis by NAC has been previously reported in neuronal,[24] lymphocyte,[23] leukemia,[26] and pancreatic β-cell derived cell lines;[27] and its mechanism of action is cell-type specific. However, it is generally assumed that the actions of NAC are due to its properties as an antioxidant or free radical scavenging agent.[23]

The underlying mechanisms for the enhancement of recombinant EPO production remain unknown. The morphological alteration of the cells shows that NAC affected cell differentiation, which might affect the rate of transcription of cellular proteins. Further studies of the molecular mechanisms of NaBu and NAC are needed in order to develop a more powerful expression system using these compounds.

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