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Butyrate-induced proapoptotic and antiangiogenic pathways in EAT cells require activation of CAD and downregulation of VEGF

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Abstract

Butyrate, a short-chain fatty acid produced in the colon, induces cell cycle arrest, differentiation, and apoptosis in transformed cell lines. In this report, we study the effects of butyrate (BuA) on the growth of Ehrlich ascites tumor (EAT) cells *in vivo*. BuA, when injected intraperitoneally (i.p) into mice, inhibited proliferation of EAT cells. Further, induction of apoptosis in EAT cells was monitored by nuclear condensation, annexin-V staining, DNA fragmentation, and translocation of caspase-activated DNase into nucleus upon BuA-treatment. Ac-DEVD-CHO, a caspase-3 inhibitor, completely inhibited BuA-induced apoptosis, indicating that activation of caspase-3 mediates the apoptotic pathway in EAT cells. The proapoptotic effect of BuA also reflects on the antiangiogenic pathway in EAT cells. The antiangiogenic effect of BuA *in vivo* was demonstrated by the downregulation of the secretion of VEGF in EAT cells. CD31 immunohistochemical staining of peritoneum sections clearly indicated a potential angioinhibitory effect of BuA in EAT cells. These results suggest that BuA, besides regulating other fundamental cellular processes, is able to modulate the expression/secretion of the key angiogenic growth factor VEGF in EAT cells.

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Angiogenesis, the growth of new capillary blood vessels from preexisting vasculature, is now being widely recognized for its role in tumor progression and metastasis [1]. Angiogenesis is a multistep process that includes endothelial cell proliferation, migration, and basement membrane degradation [1,2]. With the discovery of the role of angiogenesis in tumor growth and progression, considerable efforts have been directed towards antiangiogenic therapy as a new modality to treat human cancers. In recent years, attention has been given to identifying non-toxic, endothelial cell specific, diet-derived compounds as antiangiogenic agents for cancer therapy, in an effort to reduce the toxic side effects [3–6].

The vascular endothelial growth factor (VEGF) has been considered a key cytokine and is expressed in many

types of human malignancies including breast and ovarian cancers [7,8]. High levels of VEGF have been found in a variety of effusions accompanying pathologic disorders like edema formation in the brain, human rheumatoid synovial fluid, and malignant ascites [9]. Consequently, inhibition of fluid accumulation and tumor growth by neutralization of VEGF has been demonstrated, underlining the importance of VEGF in malignant ascites formation [10,11]. Two high affinity receptor tyrosine kinases for VEGF, VEGFR-1/Flt-1, and VEGFR-2/KDR/Flk-1, have been identified in endothelium [12]. Interaction of VEGF with Flk-1 is a requirement for malignant ascites formation [9]. Interference with the angiogenic ligand, VEGF, or its receptors, Flt-1 and KDR, offers a potential therapeutic window for treatment of malignant ascites formation and tumor growth.

Sodium butyrate, a short-chain fatty acid (SCFA) produced during the fermentation of dietary fiber by

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endogenous intestinal bacteria, is currently being evaluated as an anti-neoplastic therapeutic agent [13], and clinical trials of BuA and its derivatives in cancer patients have already been initiated [14,15]. The inhibitory role of BuA against tumor growth is mediated through a direct effect on tumor cells that results in cell cycle arrest, differentiation, or apoptosis [16–19]. BuA-induced apoptosis has been shown to involve activated caspase-3 in several cell lines [20,21]. In addition, BuA has also been shown to activate p21, a cyclin-dependent kinase inhibitor, independent of p53 activation to induce cell death [22–25]. In molecular terms, the action of BuA is probably related to histone deacetylase inhibition, leading to hyperacetylation of chromatin components, such as histones and non-histone proteins, which leads to altered gene expression [26].

In addition, BuA has been shown to modulate the activity of the transcription factor NF- κ B in a number of different cell types, including colon cancer cells [27]. The ability of BuA to modulate NF- κ B activity may arise from its ability to inhibit histone deacetylases. The antiproliferative effect of BuA on transformed cells is well established, and it has emerged as an anti-angiogenic agent by directly repressing the expression of angiogenic ligands or indirectly interfering with endothelial cell proliferation/enzymes required for angiogenesis [28–30]. We have previously shown that BuA could repress gene expression of VEGF/KDR systems in Ehrlich ascites tumor (EAT) cells in vitro [31]. Recently, it has been shown that BuA inhibits the translocation of HIF-1 α , a key regulator of VEGF, thereby inhibiting angiogenesis [32]. The molecular mechanisms involved in modulation of both apoptotic as well as antiangiogenic cascades in EAT cells (in vivo) have not been defined so far. The aim of this study was to better understand the downstream targets for BuA-induced apoptotic and/or antiangiogenic pathways during the growth of EAT cells. Here, we provide evidence that BuA induces apoptosis in EAT cells with typical features such as apoptotic bodies, DNA fragmentation, activation of caspase-activated DNase (CAD) and also, in parallel exerts antiangiogenic effects characterized by downregulation of VEGF production and endothelial cell proliferation in vivo.

Materials and methods

RNase, iodoacetate, ethidium bromide, annexin-V kit, and antibodies against CD31 were from Sigma–Aldrich, USA. Caspase-3 inhibitor Ac-DEVD-CHO, antibodies against DFF-40 (CAD), NF- κ B (FITC tagged), and ABC-reagent for CD31 staining were procured from Santa Cruz Biotechnology, CA, USA. Secondary antibodies and BCIP/NBT substrate were procured from Bangalore Genei, India. All other chemicals and reagents were of analytical grade.

EAT cell growth in vivo and butyrate treatment. EAT cells (5×10^6 cells/mouse) were injected intraperitoneally (i.p) into 8-week-old Swiss albino mice and the weights of the animals were monitored every day. Six days after tumor inoculation, 100 μ M (300 μ l/mouse) BuA was injected intraperitoneally into the EAT bearing mice every alternate day and the mice were sacrificed on the 12th day. For ELISA studies, the animals were treated from the day 2nd to 12th day of growth period.

Cell number and ascites volume. Untreated or BuA-treated EAT bearing mice were sacrificed, and small incisions were made in the abdominal region. EAT cells along with the ascites fluid were harvested into a beaker containing 2 ml saline and centrifuged at 3000 rpm for 10 min at 4 $^{\circ}$ C. Subtracting the volume of saline added previously from the volume of the supernatant gave the volume of ascites fluid. After harvesting, the EAT cells were resuspended in HBSS and counted in a hemocytometer.

Ethidium bromide/acridine orange and annexin-V staining. Nuclear staining was performed according to the method of Srinivas et al. [33]. EAT cells either untreated or treated with BuA (10 mM in vitro or 300 μ l of 100 μ M in vivo) for 2 h in complete HBSS were smeared on a glass slide, fixed with methanol/acetic acid (3:1), and air-dried in a humidified chamber. The cells were hydrated with PBS and stained with a mixture (1:1) of ethidium bromide/acridine orange (10 μ g/ml) solutions. The cells were immediately washed with PBS thrice and viewed under Leitz-DIAPLAN fluorescent microscope.

For annexin-V staining, the cells were treated as described above. In brief, EAT cells were washed with binding buffer (10 mM HEPES, pH 7.5, containing 140 mM NaCl and 2.5 mM CaCl₂) and stained with annexin-V detection kit (APO-AC) as per the protocol supplied by the manufacturer. After 10 min, cells were washed thrice with binding buffer and the greenish red apoptotic cells were observed using Leitz-DIAPLAN fluorescent microscope and photographed.

DNA isolation. EAT cells were either untreated or pretreated with Ac-DEVD-CHO (100 μ M) for 30 min at 37 $^{\circ}$ C before being treated with BuA for 2 h. The reactions were terminated using ice-cold HBSS and the supernatant was removed by centrifugation. Cells were lysed in a buffer containing 50 mM Tris-HCl, pH 8.0, and 0.5% SDS, and incubated for 30 min at 37 $^{\circ}$ C. The cell lysate was subjected to 8 M potassium acetate precipitation and left for 1 h at 4 $^{\circ}$ C. The supernatant was subjected to phenol/chloroform/isoamyl alcohol (25:24:1) extraction, followed by chloroform extraction. DNA was precipitated by adding 1:2 volumes of ice-cold ethanol. The precipitated DNA was dissolved in 50 μ l TE buffer (pH 8.0). The DNA was digested with 20 μ g/ml RNase at 37 $^{\circ}$ C for 1 h. The DNA was quantitated and resolved on 1.5% agarose gel, viewed under UV light, and documented using Uvp-BioDoc-ItTM system.

Endonuclease activity. Endonuclease activity was performed according to the method of Stein and Hansen [34] with little modification. In brief, cytosolic extracts were prepared from EAT cells either untreated or treated with BuA for 2 h in complete HBSS. After terminating the reaction with ice-cold HBSS, cells were homogenized in a glass–glass homogenizer in buffer A (10 mM Tris-HCl, pH 8.0, 0.1 mM EGTA, and 0.1% β -mercaptoethanol containing cocktail proteolytic inhibitors). Cell debris was removed by low-speed centrifugation and the remaining supernatant was centrifuged at 17,000 rpm for 1 h at 4 $^{\circ}$ C to obtain a cytosolic fraction. 1% agarose was prepared, and 10 μ g/ml ethidium bromide and 50 μ g/ml heat denatured salmon sperm DNA were added. It was then poured onto the glass petri dishes and allowed to solidify at room temperature. Wells were prepared by suction pressure and equal amounts of proteins were loaded onto each well, incubated for 12–18 h at 37 $^{\circ}$ C in a humidified chamber, and visualized under UV-transilluminator, and the lysis zone was observed around the well. The lysis of DNA in the agarose is directly proportional to activity of the endonuclease.

Western blot analysis of CAD. Twenty micrograms of nuclear extracts prepared from the cells either untreated or treated with BuA for 2 h was separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane. After blocking with 5% BSA in Tris-buffered saline,

the membrane was incubated with antibodies against CAD (DFF-40) for 2 h. The blot was washed thrice with TBST on a shaker and incubated with secondary antibody tagged to alkaline phosphatase for 1 h. The blot was washed thrice with TBST for 10 min and the CAD protein was detected by using chromogenic substrate BCIP/NBT.

VEGF-ELISA. VEGF-ELISA was carried out using the ascites of EAT bearing mice treated with or without BuA in vivo. In brief, 100 μ l of ascites from control EAT bearing mice or the BuA-treated mice was coated in a coating buffer at 4 °C overnight. Subsequently, wells were incubated with anti-VEGF₁₆₅ antibodies, followed by incubation with secondary antibodies tagged to alkaline phosphatase. BCIP/NBT was used as a substrate.

Immunohistochemistry. Excised peritoneum from the mice was either untreated or treated with BuA, was paraffin-blocked, and 5- μ m sections were prepared. The sections were fixed and immunostained for CD31 as per the protocol supplied by the manufacturer (Santa Cruz Biotechnology, CA, USA). In brief, sections were deparaffinized by using xylene and hydrated with 100% ethanol for 5 min, followed by 95% for 2 min 80% for 2 min. Subsequently, the sections were incubated with 3% H₂O₂ in PBS to block endogenous peroxidase activity. After 2 min, the sections were rinsed with PBS for 2 min, baked at 450 Hz for 10 min to retrieve the antigen, and hydrated with PBS for 2 min. Sections were incubated with anti-CD31 (PECAM-1) antibodies overnight at 4 °C. After tapping off the antibody, the slides were dipped for 5 min in PBS. The sections were incubated with secondary antibody with biotinylated rabbit anti-mouse IgG for 30 min at room temperature. The slides were washed in PBS for 5 min and incubated with ABC-reagent for 45 min. After incubation, the slides were washed with histobuffer and incubated for 10 min with the substrate, DAB peroxidase. Subsequently, the sections were counterstained with 2% hematoxylin solution for 5 min and washed in tap water thrice for 5 min each. The slides were processed and mounted using Entellan mountant solution.

Results

Antiproliferative effects of BuA on EAT cells and decreased ascites secretion in vivo

Initially, an attempt was made to understand the in vivo effect of BuA on the proliferation of EAT cells. Swiss albino mice were treated with various concentrations of BuA (0.01–10 mM). It was found that 100 μ M (300 μ l/mouse) BuA significantly showed an antiproliferative effect towards EAT cells in vivo. Upon BuA-treatment, the weight of the animals gradually decreased and the survival time of the EAT bearing mice also increased (data not shown). In contrast, the weights of untreated EAT bearing mice steadily increased and they died in normal growth period of about 12–13th day. The total number of cells in untreated mice was 13.7×10^8 whereas in BuA-treated mice there was 4.4×10^8 as shown in Fig. 1A. The significant reduction in EAT cell number in BuA-treated mice could also reflect the decreased secretion of ascites by EAT cells. The characteristic feature of EAT is that it is an ascites tumor, and in response to BuA-treatment, approximately 50% reduction in ascites secretion was observed (Fig. 1B). These preliminary data suggest that BuA acts as an antiproliferative agent in an EAT cell model system, and more importantly BuA inhibits ascites secretion in vivo.

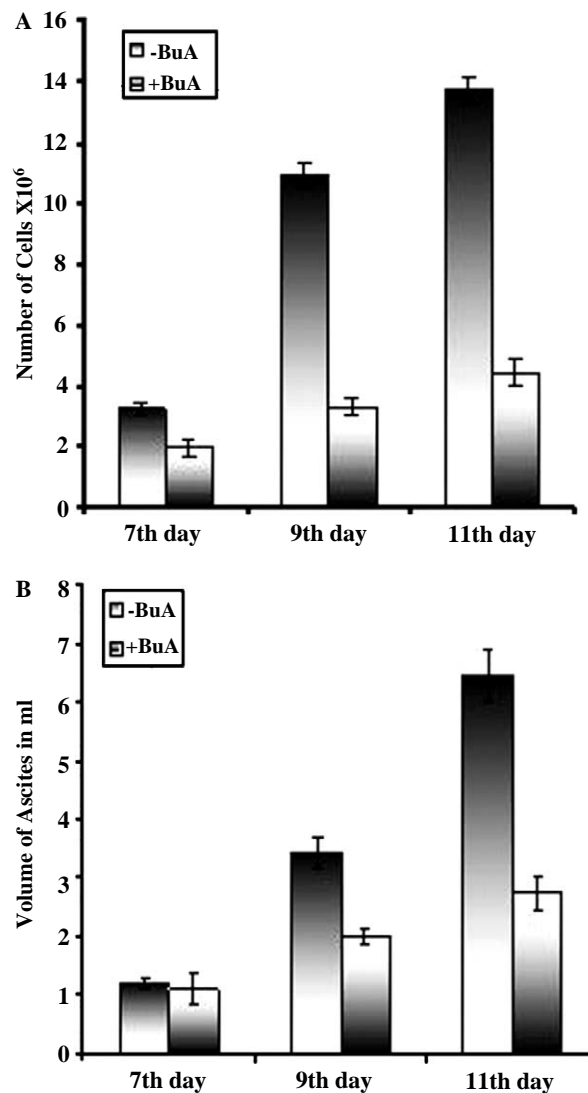


Fig. 1. (A,B) Effect of BuA on proliferation of EAT cells and ascites formation in vivo. EAT cells (5×10^6 cells/mouse, i.p) were injected into albino mice and the weights of the mice were recorded to monitor tumor growth. Every alternate day (after the 6th day of tumor inoculation) BuA was injected (100 μ M, 300 μ l/mouse) into the mice peritoneum. At least five mice were used for each experiment and the results obtained are averages of three individual experiments. Tumor-bearing mice treated with vehicle or BuA were sacrificed on 12th day. EAT cells and with ascites fluid were harvested. The total number of cells (A) and the ascites volume (B) were recorded per mouse.

BuA induces apoptosis in EAT cells

Several studies have reported that BuA induces apoptosis in a variety of cell types [20–25]. Hence, an attempt was made to identify whether the BuA-induced antiproliferative effect on EAT cells was due to induction of apoptosis. Ethidium bromide/acridine orange staining of EAT cells indicated the formation of condensed nuclei. As shown in Fig. 2A, upon BuA-treatment, most cells undergo apoptosis and showed distinct nuclear condensation as

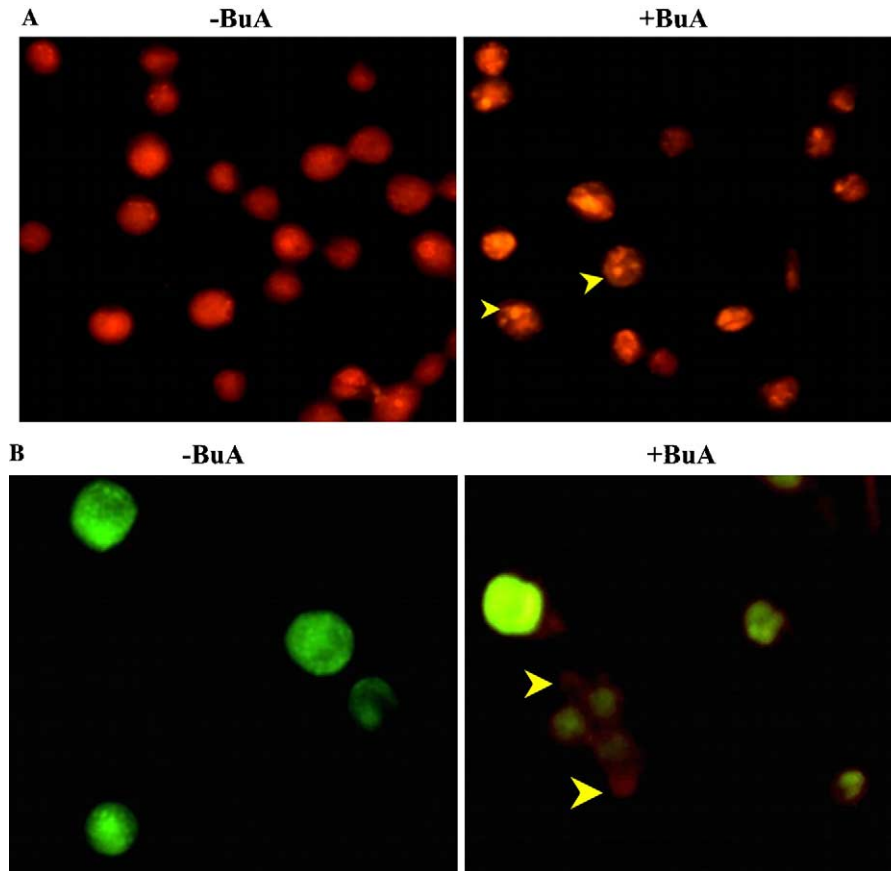


Fig. 2. Ethidium bromide/acridine orange and annexin-V staining of EAT cells. (A) EAT cells were resuspended in complete HBSS and treated with or without butyrate (10 mM, in vitro) for 2 h at 37 °C. After incubation, cells were fixed with methanol/acetic acid (3:1) on glass slides and stained with ethidium bromide/acridine orange (1:1, 10 µg/ml). Cells were washed extensively with PBS and viewed with a fluorescent microscope. (B) EAT cells were treated with BuA as mentioned above and stained using an annexin-V apoptosis detection kit as per the manufacturer's protocol.

compared to control cells which showed intact nuclear architecture. In addition, in vivo BuA-treated cells were also analyzed for nuclear condensation and showed a similar pattern as observed in in vitro BuA-treated EAT cells (data not shown).

In order to confirm that apoptosis was induced by BuA in EAT cells, we stained the cells with annexin-V. It is evident from Fig. 2B that externalization of phosphatidylserine occurred on the membrane upon BuA-treatment. In contrast, the untreated cells did not exhibit the externalization of phosphatidylserine on the membrane. The greenish red (arrows indicated) stained cells clearly confirmed the flipping of phosphatidylserine on the membrane, which is normally located inside the membrane. The externalization of phosphatidylserine could be identified in apoptotic signaling pathways, and BuA clearly showed flipping of phosphatidylserine in EAT cells, indicating the apoptotic pathway in EAT cells.

BuA induces activation of caspase-3, CAD, and endonuclease-II in EAT cells

After confirming that BuA induces EAT cell apoptosis, we next attempted to identify the mechanism of EAT

cell death. It is well recognized that various caspases play a crucial role in apoptosis. We examined whether or not BuA has any effect on the activation of caspase-3, a cysteine protease responsible for morphological changes and DNA degradation in apoptotic cells [20,21]. Preincubation of EAT cells with caspase-3 inhibitor completely inhibited BuA induced nuclear condensation (data not shown) and DNA degradation (Fig. 3A). Since BuA-induced activation of caspase-3 in EAT cells, we further verified the activation of CAD, which mediates genomic DNA degradation into nucleosomal fragmentation. Fig. 3B compares the Western blot analysis of CAD in both control and BuA-treated EAT cells. It is evident that control nuclear extract did not show the presence of CAD due to the fact that CAD is associated with its inhibitor ICAD in the cytosol [40]. Upon BuA-treatment, CAD (40 kDa) dissociated from ICAD and translocated into nucleus to fragment the genomic DNA. Consistent with the activation of caspase-3 protease in EAT cells, CAD, a downstream endonuclease, is concomitantly activated upon BuA-treatment.

Several studies have shown that activation of endonuclease II is critical for DNA fragmentation and

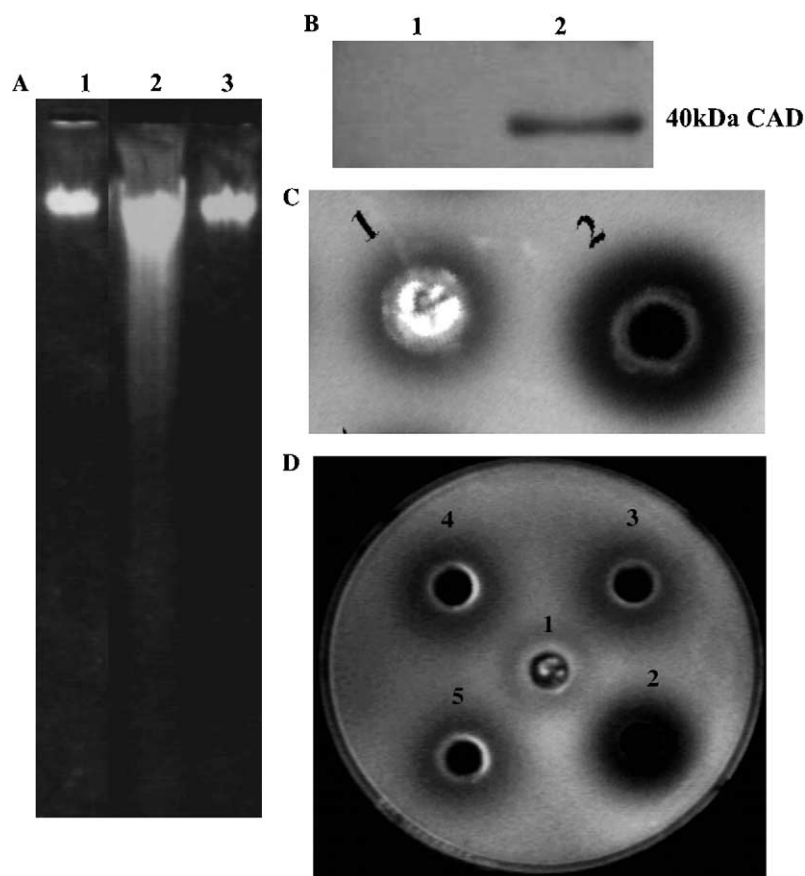


Fig. 3. Analysis of apoptotic markers in EAT cells upon BuA-treatment. (A) Effect of BuA on genomic DNA: EAT cells were either untreated or treated with BuA in complete HBSS for 2 h, and DNA was isolated by standard phenol/chloroform method. In parallel, cells were also preincubated with the caspase-3 inhibitor Ac-DEVD-CHO for 30 min (100 μ M) before being treated with BuA for 2 h and processed for DNA isolation. Ten micrograms of DNA from control as well as BuA-treated EAT cells was resolved in 1.5% agarose gel and observed using a UV-transilluminator and photographed (lane 1, control; lane 2, BuA-treated; lane 3, Ac-DEVD-CHO + BuA). (B) Activation of CAD upon BuA-treatment: 20 μ g of nuclear extract from cells either untreated or treated with BuA for 2 h was resolved by SDS-PAGE. Western blot analysis was performed using anti-CAD antibodies. Lane 1, untreated nuclear extract; lane 2, BuA-treated nuclear extract. (C) Induction of endonuclease upon BuA-treatment: cytosolic extracts were prepared from cells either untreated or treated with BuA for 2 h in complete HBSS. 1% agarose containing 10 μ g/ml ethidium bromide and 50 μ g/ml of heat-denatured salmon-sperm DNA was plated on glass petri dishes and allowed to solidify at room temperature. Wells were prepared by suction pressure and equal amounts of protein (500 μ g) from control and BuA-treated cytosolic extracts were added and incubated for 12–18 h at 37 $^{\circ}$ C in a humidified chamber. The endonuclease activity (lysis zone) was observed using a UV-transilluminator and photographed. Lane 1, untreated cytosol and lane 2, BuA-treated cytosol. (D) Effect of iodoacetate on BuA-induced endonuclease activity: EAT cells were preincubated with varying concentrations of iodoacetate (0.25–1.0 mM) for 30 min, followed by 2 h treatment with BuA at 37 $^{\circ}$ C. Cytosolic extracts were prepared as described in Materials and methods. Equal amounts of protein were loaded in each well and incubated for 12–18 h at 37 $^{\circ}$ C in a humidified atmosphere. The lysis zone for endonuclease activity was measured after visualizing with a UV-transilluminator. Lanes: 1, untreated cytosol; 2, BuA-treated cytosol; 3, 250 μ M IAA + BuA; 4, 500 μ M IAA + BuA; and 5, 1.0 mM IAA + BuA.

degradation during cell death [35–37]. Hence, an attempt was made to find out whether BuA-induced DNA degradation is associated with activation of endonuclease-II in EAT cells. As shown in Fig. 3C, when compared to the endonuclease activity of control cells where the EAT cells show basal activity, upon BuA-treatment, cells undergo apoptosis and the activation of endonuclease was clearly evident. Fig. 3D shows the inhibitor analysis of endonuclease-II, and it is obvious that preincubation of iodoacetate, an inhibitor of endonuclease-II [38,39], inhibited endonuclease-II activity in a concentration-dependent manner in BuA-treated EAT cells, suggesting the activation of endonuclease-II upon

BuA-treatment. Together, the data obtained indicate that the apoptotic pathway in EAT cells induced by BuA requires both activation of CAD and endonuclease-II for DNA fragmentation.

BuA inhibits VEGF production in EAT cells

Since BuA inhibited ascites secretion in vivo, its effect on VEGF secretion was also studied. Ascites were collected from control as well as BuA-treated mice for a period of 12 days and ELISA studies were performed. The data obtained from ELISA studies indicated that BuA has a dose-dependent effect on secretion of VEGF under in vivo

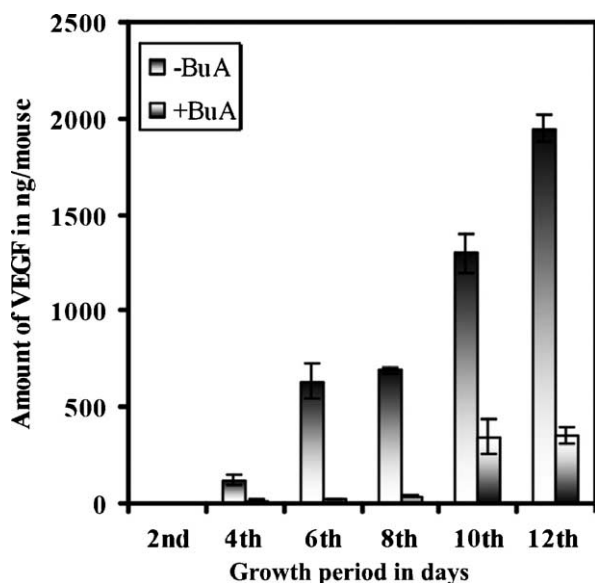


Fig. 4. Effect of BuA on in vivo production of VEGF. EAT bearing mice were injected with 100 μ M (300 μ l) BuA every alternate day and the ascites volume was collected after sacrificing the animal. The production of VEGF in EAT cells was quantitated up to the 12th day of the EAT cell growth period. ELISA was carried out to quantitate the VEGF using anti-VEGF₁₆₅ antibodies.

conditions. Fig. 4 compares the amount of VEGF at different growth period for untreated and BuA-treated EAT cells. The amount of VEGF increased in untreated EAT cells over the growth period, whereas the amount of VEGF in the ascites of BuA-treated EAT cells did not show any significant increase in synthesis over the same growth period, suggesting a dose-dependent inhibition of VEGF secretion upon BuA-treatment in EAT cells.

BuA inhibits tumor-induced neo-vascularization in EAT cells

In order to provide further evidence for the in vivo effect of BuA on VEGF production, we looked to verify its effect on endothelial proliferation in the peritoneum of EAT-bearing mice. Fig. 5 shows the comparison between the immunohistochemical staining of peritoneum sections from control and BuA-treated EAT cells. H&E staining of peritoneum sections from the control group appeared well vascularized. In contrast, BuA-treated peritoneum sections were characterized by a pronounced decrease in vascularity (Fig. 5A), both in terms of microvascular density (MVD) and the caliber of the detectable vascular channels. Control peritoneum sections showed 20 ± 3.3 blood vessels whereas in case of BuA-treated peritoneum, only 9.1 ± 2.2 blood vessels were observed. Furthermore, staining for the endothelial-specific marker CD31 showed a striking difference between control and BuA-treated EAT cells. It is evident that an appreciable

amount of endothelial staining (CD31) and infiltrating tumor cells were observed in control peritoneum sections (Fig. 5B). In contrast, BuA-treated peritoneum sections showed a significant reduction in endothelial staining and tumor infiltration over the experimental period. These results demonstrate that the apoptotic effects of BuA on EAT cells could also affect the proliferation of endothelial cells and blood vessel formation by inhibiting VEGF secretion.

Discussion

The short-chain fatty acid BuA is known to induce apoptosis and differentiation of cancer cells [22]. In addition, most of the recent studies on BuA suggest that it is an antiangiogenic agent [23,24]. We have shown previously that BuA can repress the gene expression of the angiogenic ligand VEGF and its receptor KDR in vitro [31]. In this report, we have focused our attention on delineating the precise molecular mechanism underlying the proapoptotic and antiangiogenic activity of BuA in vivo in EAT cells.

We demonstrate an inhibition of proliferation of EAT cell growth in vivo with a corresponding reduction in ascites volume and cell number upon BuA-treatment. Several studies have shown that BuA can induce apoptosis in variety of transformed cell types [18,19]. Consistent with other studies, our results show that there is an induction of apoptosis in EAT cells upon BuA-treatment. The morphological features of apoptosis such as nuclear condensation and fragmentation of genomic DNA were clearly indicative in BuA-treated EAT cells. Caspase proteases play a crucial role in inducing apoptosis, and BuA has been shown to induce the activation of caspases during apoptosis [20,21]. Our studies demonstrate that Ac-DEVD-CHO, a specific caspase-3 inhibitor, completely inhibited BuA-induced apoptosis, suggesting that BuA-induced apoptosis in EAT cells is mediated via the caspase-3 pathway. Activation of caspase-3 has been shown to act on downstream targets such as the DNA-repair enzyme poly (ADP-ribose) polymerase (PARP) or DNA fragmentation factor (DEF), leading to the breaking of DNA strands into 180 bp pieces [20,41]. It is evident from our results that BuA can activate CAD, which is associated with its inhibitor ICAD in the cytosol [42]. Several studies have shown that activation of endonuclease II is required for DNA fragmentation and degradation during cell death [35–37,43]. Our results clearly demonstrate that activation of endonuclease-II upon BuA-treatment, which supports our view that BuA could activate both CAD and DNase-II for complete DNA degradation in EAT cells, and thus suggest that apoptotic pathway induced by BuA in EAT cells is at least in part mediated by the activation of CAD and DNase-II.

In parallel observation, the proapoptotic action of BuA on EAT cells reflects on decreased levels of secretion

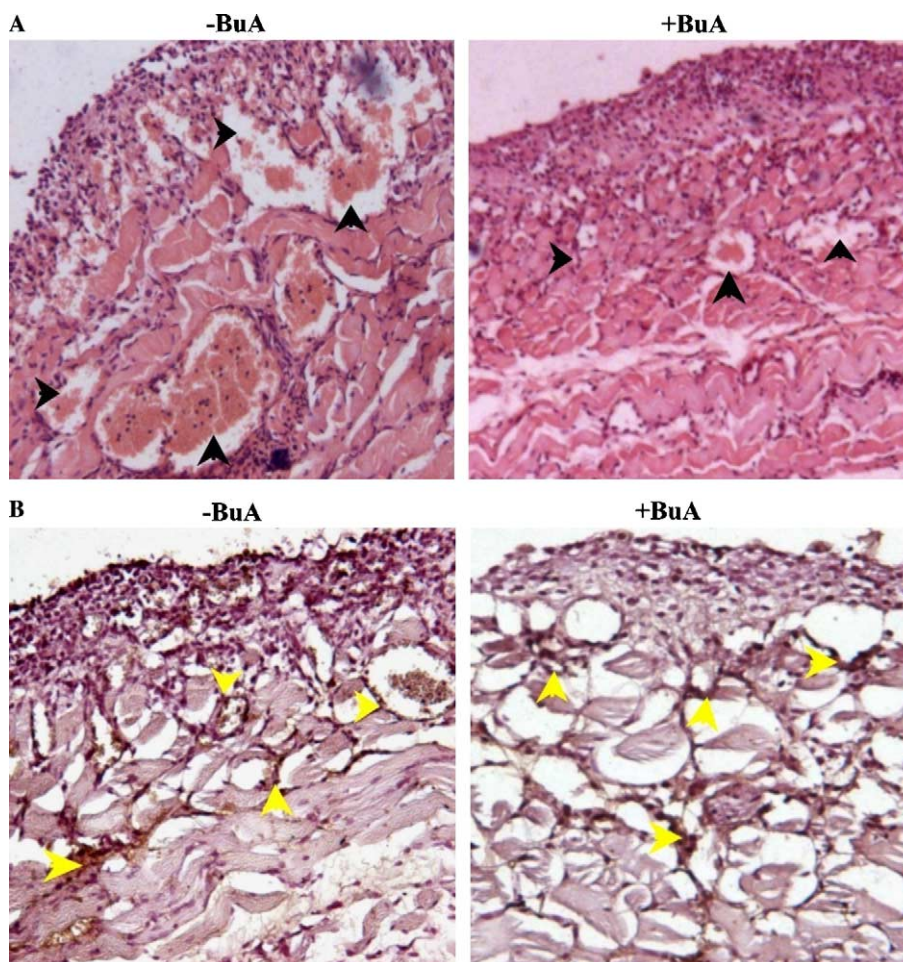


Fig. 5. Immunohistochemical studies of EAT bearing mouse peritoneum. (A) H&E staining of peritoneum sections of the EAT bearing mice either untreated or treated with BuA in vivo. The arrows indicate the blood vessels in the peritoneum. (B) CD31 staining of control and BuA-treated EAT bearing mouse peritoneum. Sections from the peritoneum that were either untreated or BuA-treated were incubated with anti-CD31 antibodies overnight at 4 °C and stained with ABC-reagent according to the manufacturer's protocol. The arrows indicate the presence of endothelial cell lining and infiltrating tumor cells in the peritoneum of the mouse.

of ascites and VEGF production. The antiangiogenic effect of BuA on endothelial cells has been shown to act on COX-2 expression [30]. Similarly, studies have also shown to inhibit the translocation of HIF-1 α transcription in response to BuA-treatment [32]. We and others have previously shown the direct effect of BuA on VEGF and its receptors [31,28,29]. Consistent with the previous reports, our data demonstrate a dose-dependent inhibition of VEGF secretion in vivo. The in vivo effect of BuA on proliferation of endothelial cells is especially visible when observing blood vessel formation in the peritoneum of mice. The immunohistological studies clearly show the inhibition of blood vessel formation in H&E staining and CD31 analysis, and thus suggest that BuA primarily acts on endothelial cells, and hence inhibition of angiogenesis. The biphasic effect of BuA as proapoptotic and antiangiogenic compound is consistent with those of some other anticancer drugs [43,44] such as cyclophosphamide [45], and camptothecin [46,47], thus strongly

suggesting that these kinds of anti-tumor compounds seem to be more promising in clinical practice than the compounds that directly target angiogenesis.

In summary, the data presented here provide evidence that BuA could act as both proapoptotic as well as an antiangiogenic agent in EAT cells. The multiple beneficial effects of BuA on EAT cell growth indicate that this model system would be valuable for use in developing alternative therapeutic approaches in the treatment of human ascites tumor malignancies.

Acknowledgments

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