Induction of Apoptosis by Arachidonic Acid in Chronic Myeloid Leukemia Cells

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ABSTRACT

The hallmark of chronic myeloid leukemia (CML) is the presence of the bcr-abl oncogene, which is associated with transforming ability and an intrinsic resistance to induction of apoptosis by genotoxic agents. Arachidonic acid (AA), a biologically active fatty acid, plays a crucial role as a mediator of signaling pathways involved in cell proliferation and survival. In this study, we investigated the potential role of AA as a proapoptotic agent in CML. Pretreatment of human CML isolated progenitor cells with AA (100 μM for 18 h) induced 71–75% inhibition of in vitro colony formation of granulocyte-macrophage colony-forming units, multilineage colony-forming units, and erythroid burst-forming units. This inhibition was significantly greater than the effect on normal progenitor cells (19–39% growth inhibition of erythroid burst-forming units, multilineage colony-forming units, and granulocyte-macrophage colony-forming units). AA also inhibited growth of the bcr-abl-transformed cell line H7.bcr-abl A54. In contrast, a minimal effect of AA on inhibition of cell growth was observed in the parental nontransformed NSF/N1.H7 cell line. The antiproliferative effect of AA was associated with apoptosis. γ-Linolenic acid, a precursor of AA, also inhibited cell growth, whereas other unsaturated and saturated fatty acids had no effect. Pharmacological inhibition of cyclooxygenase, lipoxygenase, and cytochrome P450 monooxygenase enzymes prior to exposure to AA did not rescue cells from the inhibitory effect of AA. Moreover, 5,8,11,14-eicosaetraynoic acid, a nonmetabolizable arachidionate analogue, also inhibited cell growth, suggesting that the effect of AA did not require further metabolism. Treatment with antioxidants prior to stimulation with AA was also ineffective in preventing its antiproliferative effect. Thus, AA inhibited proliferation of CML cells by inducing apoptotic cell death. The signaling mechanisms of AA-induced inhibition of cell growth appeared to be independent of its conversion into eicosanoids or free radical generation.

INTRODUCTION

CML is a disorder characterized by a massive expansion of progenitor cells in all stages of maturation (1). CML is associated with the Philadelphia chromosome (Ph), a cytogenetic abnormality generated by the reciprocal translocation between the bcr gene on chromosome 22q and the c-abl proto-oncogene on chromosome 9q (1). The bcr-abl fusion gene codes a M₄ 210,000 fusion protein (p210 bcr-abl) with transforming activity for hematopoietic cells and the ability to cause CML-like myelopoeisis in mice (2, 3). Bcr-abl signaling causes transformation through several mechanisms (4, 5). Earlier studies have postulated that the altered tyrosine kinase activity of p210 bcr-abl stimulates uncontrolled cell proliferation, leading to the massive clonal expansion of hematopoietic progenitors detected in CML (6, 7). However, subsequent studies revealed that CML progenitors have similar proliferation rates to their normal counterpart and that p210 bcr-abl increases cell survival by inhibiting apoptosis (8). Thus, p210 bcr-abl may act through an antiapoptotic mechanism in conferring a proliferative advantage to CML cells (9). Consistent with this possibility, antisense oligonucleotides targeted against bcr-abl render CML cells susceptible to the induction of apoptosis by serum deprivation or by chemotherapeutic agents (10). Furthermore, inhibition of bcr-abl kinase activity by the tyrosine kinase inhibitor, genistein, induced inhibition of cell growth associated with apoptosis (11). However, a normal reaction of CML cells to death-inducing stimuli has also been observed (12).

AA, a polyunsaturated fatty acid, is liberated from membrane phospholipids upon cell activation by a wide array of external stimuli (13, 14). Upon release, AA is rapidly converted into a number of metabolites with enhanced or altered biological activity (15). Although many effects of AA on cell function are mediated by its conversion into eicosanoids (13, 15), we and others (16, 17) have shown that AA per se is involved in the regulation of a number of signal transduction pathways. These findings suggest a role for AA as a second messenger in cell functions.

Several lines of evidence indicate that AA or its metabolites are involved in modulating growth and survival of hematopoietic cells. Anti-immunoglobulin stimulation of the immature B cell line, WEHI231, induces growth arrest and apoptosis, through activation of cytosolic phospholipase A₂ and consequent liberation of AA (18). cis-Unsaturated fatty acids, including AA, have been shown to suppress human T-cell proliferation by a free radical-dependent process (19). On the contrary, AA induces apoptosis in HL-60 cells by a mechanism that is independent of free radical generation and eicosanoids (20). Human neutrophils are also susceptible to the apoptotic effect of AA (21). Certain products of AA metabolism have been implicated as positive modulators of hematopoietic cell growth. Thus, transfection of W256 carcinosarcoma of hematopoietic origin with antisense oligonucleotides directed against 12-lipoxygenase induced growth arrest and apoptosis (22). Furthermore, pharmacological inhibition of 5-lipoxygenase pathway by MK886 inhibited proliferation and induced apoptosis in the monocytic cell line U937 and in MNCs isolated from peripheral blood of a patient with chronic-phase CML (23). These findings suggest that lipoxygenase products of AA metabolism are involved in supporting proliferation and survival of leukemic cells.

Information on the effect of AA on CML cell proliferation is lacking. The findings described here are the first to demonstrate an effect of AA on highly purified immature progenitor cells from CML and normal marrow. We present evidence that AA induces inhibition of cell growth and apoptosis in CML progenitor cells as well as in bcr-abl-transformed cell lines. Furthermore, our studies suggest that the signaling pathway of AA-induced inhibition of cell growth is independent of generation of eicosanoids or free radicals.

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3 The abbreviations used are: CML, chronic myeloid leukemia; AA, arachidonic acid; MNC, mononuclear cell; FBS, fetal bovine serum; ETYA, 5,8,11,14-eicosatetraynoic acid; NDGA, nordihydroguaiaretic acid; TGT, terminal deoxynucleotidyl transferase; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; SCF, stem cell factor; CFU-Mix, multilineage colony-forming units; BFU-E, erythroid burst-forming units; CFU-GM, granulocyte-macrophage colony-forming units; LTC, long-term culture; LTC-IC, LTC-initiating cell; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.
MATERIALS AND METHODS

Reagents. McCoy’s conditioned medium, RPMI 1640, and Iscove’s medium were purchased from Life Technologies, Inc. (Gathersburg, MD). Iscove’s modified Dulbecco’s medium was purchased from Seromed (Berlin, Germany). FBS was purchased from Hyclone (Logan, UT). AA, oleic acid, linoleic acid, linolenic acid, palmitic acid, desferrioxamine, E'TYA, vitamin E, indomethacin, caffeic acid, NDGA, and ketoconazole were obtained from Sigma Chemical Co. (St. Louis, MO). γ-Linolenic acid was purchased from Cayman (Ann Arbor, MI). Catalase was purchased from Calbiochem (San Diego, CA). The nonradioactive proliferation assay was obtained from Promega (Madison, WI). The TdT kit and DNA molecular weight markers were purchased from Boehringer Mannheim (Indianapolis, IN). IL-3 and G-CSF were obtained from Sandoz (Basel, Switzerland). GM-CSF, erythropoietin, and SCF were obtained from Amgen (Thousand Oaks, CA).

Cell Lines. The characteristics of the IL-3-dependent murine myeloid cell line, NSF/N1.H7, and the p210c-abl stable-transformed derivative cell line, H7.bcr-abl A54, have been described elsewhere (24). NSF/N1.H7 cells were maintained in McCoy’s medium containing 10% WEHI-conditioned medium, as a source of IL-3, and FBS (10%, v/v). H7.bcr-abl A54 cells were maintained in McCoy’s medium containing FBS (10%, v/v) only and were routinely treated with G418 (250 μg/ml) to prevent additional random mutations.

Bone Marrow and Peripheral Blood Cells. CML marrow cells were obtained by aspiration from the posterior iliac crest. Four patients (two males and two females) with median age of 51 years (range, 40–60 years) and a diagnosis of Ph-positive CML in chronic phase were included in this study. All patients were studied at diagnosis prior to any treatment and were 100% Ph-positive at direct cytogenetic analysis. Three patients showed a b3a2 bcr-abl junction, and 1 case showed a b2a2 junction by reverse transcriptase-PCR analysis. Normal cells were obtained from healthy donors undergoing peripheral blood progenitor cell mobilization. All patients and normal individuals provided informed consent for these studies.

Cell Separation Procedures. MNCs were separated by centrifugation on Ficoll-Hypaque (11). MNCs were washed and resuspended in RPMI 1640 supplemented with 10% FBS. CD34+ cells were enriched according to a magnetic cell sorting methodology (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany; Ref. 25). The purities of CML and normal CD34+ cell fractions ranged from 63 to 97% and from 75 to 86%, respectively (25).

CFU-Mix, BFU-E, and CFU-GM Assays. The assays for CFU-Mix, BFU-E, and CFU-GM were carried out as described elsewhere (11, 26). Briefly, 1 × 10⁶ CD34+ cells were plated in 35-mm Petri dishes in 1 ml aliquots of Iscove’s modified Dulbecco’s medium containing 30% FBS, 10⁻⁴ M β-mercaptoethanol, and 1.15% (w/v) methylcellulose. Cultures were stimulated with SCF (10 ng/ml), IL-3 (10 ng/ml), GM-CSF (10 ng/ml), G-CSF (10 ng/ml), and Epo (3 units/ml). Cell growth was evaluated after incubation (37°C, 5% CO2) for 14–18 days in a humidified atmosphere. Four dishes were set up for each individual data point per experiment. Colonies were scored according to previously published criteria (26).

LTC-IC Assay. The LTC-IC assay was performed as described previously (11, 23). Briefly, CD34+ cells (1 × 10⁴) were seeded into cultures containing a feeder layer of irradiated (8000 cGy) murine M2–10B4 cells (3 × 10⁵/cm²), kindly provided by Dr. C. Eaves, Terry Fox Laboratory, Vancouver, British Columbia, Canada) engineered to produce human IL-3 and human G-CSF (27). After 5 weeks in culture, nonadherent and adherent cells were harvested and assayed together for clonogenic cells in standard methylcellulose cultures stimulated with IL-6 (10 ng/ml), SCF (50 ng/ml), IL-3 (10 ng/ml), G-CSF (10 ng/ml), GM-CSF (10 ng/ml), and Epo (3 units/ml). The total number of clonogenic cells present in 5-week-old LTCs provides a relative measure of the number of LTC-ICs originally present in the test suspension (28). Absolute LTC-IC values were calculated by dividing the total number of clonogenic cells by 4, which is the average output of clonogenic cells per LTC-IC, according to limiting dilution analysis studies reported by others (28).

DNA Electrophoresis. Cells (1 × 10⁷/ml) were treated with either vehicle or AA for the indicated times. DNA laddering was detected by isolating fragmented DNA using the SDS/proteinase K/RNase extraction method, which allows the isolation of only fragmented DNA without contaminating genomic DNA (29). Briefly, cells were washed in cold PBS and lysed in a buffer containing 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), 0.5% SDS, and proteinase K (0.5 mg/ml) at 50°C for 1 h. RNase A (0.2 mg/ml) was added, and incubation continued for an additional hour at 50°C. Samples were diluted 2:1 with loading buffer (10 mM EDTA, 1% agarose, 0.25% bromphenol blue, and 40% sucrose) heated at 70°C for 10 min and subjected to electrophoresis on 2% agarose gel. DNA bands were visualized under UV light after staining with ethidium bromide.

Detection of Apoptosis by the TdT-mediated UTP Nick End-Labeling Assay. Cells (1 × 10⁶/ml) were treated with AA or vehicle as indicated. DNA strand breaks generated during apoptosis were identified by labeling free 3’-OH termini with fluorescein-dNTP in the presence of TdT, as described previously (11). Cells were analyzed by flow cytometry on a FACScan (Becton Dickinson) following incubation for 60 min at 37°C.

Cell Proliferation Assay. Cell proliferation was evaluated by the colorimetric MTS assay with the electron coupling reagent p-nitrophenyl methosulfate, according to the manufacturer’s protocol. Briefly, cells (1 × 10⁶/ml) were seeded in 24-well plates and subsequently stimulated with various concentrations of AA for 24–72 h in the presence of 10% FBS. At the times indicated, 0.2 ml of MTS/p-nitrophenyl methosulfate mixture was added to 1 ml of cell suspension and allowed to incubate for 2 h at 37°C and 5% CO₂ before absorbance at 490 nm was measured. Background absorbance from medium only was subtracted from each experimental condition.

Transmission Electron Microscopy. Vehicle- or AA-treated cells were washed, pelleted, and fixed with 2.5% glutaraldehyde in 100 mM phosphate buffer (pH 7.4) for 2 h at room temperature. Samples were prepared for transmission electron microscopy, dehydrated, and embedded in Epon. Grids were stained with uranyl acetate and Reynolds’s lead and examined at 80 keV using a Phillips EM-201.

Statistical Analysis. For the in vitro growth of human isolated progenitor cells, experiments were performed in quadruplicate, and the results were expressed as mean ± SD. Statistical analysis was performed with the Statview software using a Macintosh 6300 personal computer (Apple Computer Inc., Cupertino, CA). The Wilcoxon signed-rank test was used to assess significance of changes in the incidence of progenitor cell growth from untreated and AA-treated cells. For the proliferation assay, experiments were performed in triplicate, and results were expressed as mean ± SD.

RESULTS

Effect of AA on Immature Progenitor Cell Growth from CML Marrow. Preliminary experiments to define the optimal concentrations of AA capable of inhibiting colony formation from CD34+ isolated progenitor cells showed that the maximum inhibitory effect was observed following preincubation with AA at a concentration of 100 μM in medium containing 10% FBS (data not shown). AA affected the growth pattern of CD34+ derived-CFU-Mix, CFU-GM, and BFU-E colonies in a time-dependent manner. Compared with cells treated with vehicle only, exposure of CML CD34+ cells (1 × 10⁵/ml, n = 4) to 100 μM AA for 1 and 2 h, following by extensive washings, induced a statistically significant (P = 0.046) inhibition of progenitor cell growth, which ranged from 31 to 40% (Fig. 1A). After 18 h of preincubation with AA (100 μM), growth of CFU-Mix, BFU-E, and CFU-GM was inhibited by 77% (P = 0.042), 75% (P = 0.042), and 71% (P = 0.027), respectively (Fig. 1A). However, when normal CD34+ cells (n = 5) were preincubated with AA (100 μM) for 1 and 2 h, no inhibition on the growth of CFU-Mix, BFU-E, and CFU-GM was detected (Fig. 1B). Indeed, there was an apparent increase in the mean colony growth in normal CFU-Mix after 1 h exposure to AA, but this increase did not reach statistical significance (P = 0.46). Following 18 h of preincubation, AA suppressed CFU-Mix, BFU-E, and GFU-GM by 39% (P = 0.046), 19% (P = 0.34), and 34% (P = 0.046), respectively (Fig. 1B).

The effect of AA on the more primitive LTC-IC progenitors was also investigated. The percentages of CML LTC-ICs surviving 1, 2, and 18 h exposure to AA (100 μM) were 105% (P = 0.65), 89% (P = 0.28), and 48% (P = 0.044), respectively (Fig. 2). Under the same experimental conditions, the number of normal LTC-IC surviving pretreatment with
AA were 101% (P = 0.1), 123% (P = 0.28), and 76% (P = 0.28) at 1, 2, and 18 h, respectively (Fig. 2). Thus, 18 h of exposure of CML LTC-ICs to AA induced a significant inhibition of cell growth (Fig. 2).

**Effect of AA on Growth of bcr-abl-transformed Cell Lines.** The effect of AA was next evaluated in the bcr-abl-expressing cell line H7.bcr-abl A54. Cells were incubated with various concentrations of AA at concentrations between 10 and 50 μM for 72 h. Indeed, NDGA and ketoconazole enhanced the inhibitory effect of AA on cell growth (Fig. 8). Thus, equimolar concentrations of AA (100 μM) were more effective in inducing inhibition of bcr-abl-transformed cells compared with the parental cells.

**Effect of AA on Apoptosis.** The results described above led us to postulate that inhibition of cell growth induced by AA might be a result of apoptotic cell death. To investigate this possibility, we examined the morphology of H7.bcr-abl A54 cells by transmission electron microscopy prior to or after treatment with AA. After 24 h treatment with AA (50 μM), the percentage of H7.bcr-abl A54 cells showing morphological features characteristic of the apoptotic mode of cell death (30) was 38 ± 0.3%. An extensive cytoplasmic vacuolization, modest signs of chromatin condensation, and nuclear membrane blebs were observed in cells treated with AA (Fig. 4A) but not in vehicle-treated cells (<1%; Fig. 4B).

We next performed experiments to determine whether AA triggered apoptosis of CD34+ cells. CD34+ derived from CML cells were exposed to AA (100 μM for 18 h) or to vehicle in medium containing 10% FBS. Cells were permeabilized, labeled with dUTP, and subjected to FACS analysis for detection of apoptotic cells. Upon treatment with AA, 35% of CD34+ CML cells underwent apoptosis, as compared with cells treated with vehicle only (Fig. 5). We also used agarose gel electrophoresis to detect DNA fragmentation of CML CD34+ cells exposed to AA. Treatment of cells with AA (100 μM, 18 h) induced DNA fragmentation (Fig. 6, Lane 3), which was absent in vehicle-treated cells (Fig. 6, Lane 2).

**Specificity of AA-induced Inhibition of Cell Growth.** The effect of AA on cell growth was compared with that of other essential and nonessential fatty acids with various degree of unsaturation to determine its specificity. Of the fatty acids tested, γ-linoleic acid, a C-18 precursor of AA, displayed a marked inhibitory effect on H7.bcr-abl A54 cell proliferation. In contrast, the polyunsaturated oleic acid, linoleic acid, and linolenic acid and the saturated palmitic acid had a minimal effect on proliferation of H7.bcr-abl A54 cells (Fig. 7).

To clarify whether the effect of AA on cell proliferation was mediated by its conversion into eicosanoids, we examined whether inhibitors of cyclooxygenase, lipooxygenase, and cytochrome P450 monoxygenase enzymes prevented the cytotoxic effect of AA. Preincubation for 30 min with 10 μM indomethacin, a specific cyclooxygenase inhibitor, followed by stimulation with AA (50 μM for 72 h) had no effect on cell proliferation (Fig. 8). NDGA (an inhibitor of 5-, 12-, and 15-epoxyeicosanoic acid, caffeic acid (an inhibitor of 5-lipoxygenase), and ketoconazole (an inhibitor of the cytochrome P450 monooxygenase) system did not block AA-induced inhibition of cell growth (Fig. 8). Indeed, NDGA and ketoconazole enhanced the inhibitory effect of AA on cell growth, 100 μM AA caused 34% inhibition of cell growth (Fig. 3B). Thus, equimolar concentrations of AA (100 μM) were more effective in inducing inhibition of bcr-abl-transformed cells compared with the parental cells.

![Fig. 1](image1.png) **Fig. 1.** Effect of preincubation with AA on CML (A) and normal (B) CD34+ derived CFU-Mix, BFU-E, and CFU-GM. CML or normal CD34+ cells (1 × 10^6/ml) were incubated with AA (100 μM) for 1 (a), 2 (b), and 18 (c) h; washed; and cultured in methylcellulose. Columns, mean percentages of colonies growing in AA-treated samples as compared with untreated control samples; bars, SE. Four separate experiments were performed using different marrow samples obtained from consenting CML patients at diagnosis. Control colonies per 1 × 10^6 CD34+ cells ranged from 25 to 350 for CFU-Mix, from 600 to 1750 for BFU-E, and from 1200 to 7250 for CFU-GM. Five separate experiments were performed.

![Fig. 2](image2.png) **Fig. 2.** Effect of preincubation with AA on normal and CML CD34+ derived LTC-IC. CD34+ cells (1 × 10^6/ml) were incubated with AA (100 μM) for 1, 2, and 18 h; washed; and assayed in a LTC system. Columns, mean percentages of colonies growing in AA-treated samples as compared with untreated control samples; bars, SE. Four separate experiments were performed using different normal blood or CML marrow samples. Control LTC-ICs per 2 × 10^6 CD34+ cells ranged from 767 to 12,000 for normal mobilized blood and from 96 to 1,125 for CML marrow. *, statistically significant (P ≤ 0.05) as compared with control cultures (Wilcoxon signed-rank test).
from a representative experiment performed in triplicate; bars representative of three experiments; subtracted from each experimental sample. Background absorbance from medium only and from cells plated at time zero was determined at 72 h by the MTS assay. Absorbance was measured at 490 nm, and 24-well plates were stimulated with the indicated concentrations of AA. Cell proliferation was measured by the MTS assay after 72 h of incubation (Fig. 9). The degree of ETYA-induced inhibition of cell growth in a dose-dependent manner (Fig. 10). The selective involvement of lipid peroxidation of cell membrane and generation of reactive oxygen radicals. To test this possibility, we assayed a series of antioxidants including vitamin E, catalase, and desferrioxamine for their ability to overcome the inhibitory effect of AA on cell growth. H7.bcr-abl A54 cells were preincubated with vitamin E (200 μM), catalase (1000 units/ml), or desferrioxamine (0.3 mM) following stimulation with 50 μM AA. Cell growth was evaluated at 72 h by the MTS assay. None of the antioxidants tested were able to block AA-dependent inhibition of cell growth (Fig. 10). In addition, vitamin E and catalase alone were ineffective, whereas desferrioxamine induced inhibition of cell growth in the absence of AA (Fig. 10).

**DISCUSSION**

Apoptosis is a highly regulated process that is involved in physiological as well as in pathological conditions (32–34). Decreased susceptibility to respond to apoptotic stimuli has been associated with oncogenic transformation (34).

Recent studies have elucidated the involvement of lipid signaling pathways in apoptotic cell death (18, 35). Ceramide, a sphingolipid that is postulated to play a potential role as a second messenger, induces apoptosis in human CML progenitor cells (35, 36). Our previous investigations demonstrated a role for AA as a modulator of intracellular pathways potentially involved in cell proliferation and survival. (37, 38). Several studies have shown that AA either per se or after conversion into eicosanoids regulates cell growth and survival in different cell types (39, 40). However, information on the effect of AA on proliferation and survival of CML cells is fragmentary, and only indirect evidence implicates AA in apoptosis of CML cells (41). In this study, we provide evidence for a critical role of AA in inhibition of cell growth and induction of apoptosis in primitive and committed progenitors from primary CML marrow and in bcr-abl-transformed cell lines. Thus, we showed that the colony formation ability of CD34+ cells was severely impaired by pretreatment with AA in a dose- and time-dependent fashion. An intriguing observation emerging from this study was that AA significantly inhibited growth of CML CD34+ cells as compared with normal CD34+ cells. Similarly, the bcr-abl-transformed cell line H7.bcr-abl A54 was more sensitive to AA than the nontransformed counterpart NSF/N1.H7 cells. These findings suggest that leukemic cells are more susceptible to the antiproliferative effect of AA than normal cells. This selective inhibitory effect of AA on tumor cells has been observed in other cell systems. Thus, treatment of human gliomas with AA-induced apoptosis of tumor cells, whereas normal cells were less sensitive (42). Similarly, AA induces apoptosis of a human SV40-transformed lymphoblast cell line but not of the nontransformed cells (43). The mechanisms underlying the selectivity of the effect of AA toward neoplastic cells remain elusive. Studies by Begin et al. (44) showed that certain tumor cells had low levels of p210bcr-abl itself, Shc, and Cbl in the absence of AA, inhibited cell growth by 19.6 and 24.3%, respectively (data not shown).

ETYA is a structural analogue of AA in which four alkene bonds replace the four alkane bonds present in AA (31). Therefore, ETYA acts as a false substrate for the cyclooxygenase and lipoxygenase enzymes and, thus, as a nonmetabolizable analogue of AA (31). We used ETYA to confirm that the effect of AA on cell growth was not mediated by its metabolites. H7.bcr-abl A54 cells were preincubated with various concentrations of ETYA (10–100 μM) in medium containing serum (10%). Cell proliferation was measured by the MTS assay after 72 h of incubation. Treatment with ETYA inhibited cell growth in a dose-dependent manner (Fig. 9). The degree of ETYA-induced inhibition of cell growth was similar to that induced by AA (see Fig. 4A).

**Lack of Involvement of Reactive Oxygen Species in Arachidonate-induced Inhibition of Cell Growth.** To elucidate the signaling pathway by which AA induces apoptosis, we investigated the potential involvement of lipid peroxidation of cell membrane and generation of reactive oxygen radicals. To test this possibility, we assayed a series of antioxidants including vitamin E, catalase, and desferrioxamine for their ability to overcome the inhibitory effect of AA on cell growth. H7.bcr-abl A54 cells were preincubated with vitamin E (200 μM), catalase (1000 units/ml), or desferrioxamine (0.3 mM) following stimulation with 50 μM AA. Cell growth was evaluated at 72 h by the MTS assay. None of the antioxidants tested were able to block AA-dependent inhibition of cell growth (Fig. 10). In addition, vitamin E and catalase alone were ineffective, whereas desferrioxamine induced inhibition of cell growth in the absence of AA (Fig. 10).

**Fig. 3.** A, effect of AA on growth of H7.bcr-abl A54 cells. Cells (1 × 10^5/ml) in 24-well plates were stimulated with the indicated concentrations of AA. Cell proliferation was determined at 72 h by the MTS assay. Absorbance was measured at 490 nm, and background absorbance from medium only and from cells plated at time zero was subtracted from each experimental sample. Columns, mean absorbance units from a representative of three experiments; bars, SD. B, effect of AA on growth of NSF/N1.H7 cells. Cells were stimulated with AA under the same conditions described above. Cell proliferation was evaluated at 72 h by the MTS assay. Columns, mean absorbance units from a representative experiment performed in triplicate; bars, SD.

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Accelerates ceramide-induced apoptosis, possibly by activating additional downstream effectors (36). The potential effect of AA on bcr-abl activity was not investigated here. Thus, it is possible that a similar mechanism may explain arachidonate sensitivity of bcr-abl-transformed cells. Further investigations are underway to explore this possibility.

Inhibition of cell growth by AA was associated with induction of apoptosis in primary CML and bcr-abl-transformed cells, as determined by morphological examination, detection of DNA ladder formation, and analysis of fluorescent end-labeling DNA breaks.

Several lines of evidence have suggested that metabolites of AA are involved in control of cell growth. Prostaglandins play a role as positive regulators of cell growth in cancer cells, and overexpression of the Cox2 gene has been associated with inhibition of apoptosis (46). On the other hand, prostaglandin E2 has been implicated in induction of apoptosis in immature normal and malignant lymphocyte (47). Products of 5-, 12-, and 15-lipoxygenase also positively modulate cell growth in different cell lines. Thus, an elevated production of LTC4 and an increased activity of LTC4 synthase were detected in myeloid cells from CML patients, suggesting an autocrine production of this metabolite in maintaining leukemic cell growth (48). On the other hand, lipoxygenase products of AA metabolism play a role in tumor necrosis factor-α-induced toxicity of L929 cells and 15-hydroxyeicosatetraenoic acid (15-HPETE) induces apoptosis in HIV-infected T cells (49, 50). The results presented here showed that pharmacological inhibition of AA metabolism did not rescue the inhibitory effect of AA on cell growth, suggesting that the antiproliferative effect of AA is not mediated by its conversion into eicosanoids. Consistent with this possibility, ETYA had a cytotoxic effect on H7.bcr-abl A54 cells. Indomethacin and caffeic acid did not increase AA-induced inhibition of cell growth, whereas NDGA and ketoconazole did. Further-

Fig. 4. Morphological changes induced by AA in H7.bcr-abl A54 cells. A, cells (1 × 10⁶/ml) were treated with AA (50 μM) for 24 h, harvested, fixed in 2.5% glutaraldehyde, and analyzed by electron microscopy. Arrows, cytoplasmic vacuoles and nuclear membrane blebs. B, vehicle-treated cells (original magnification, ×12,600). Shown are cells from a representative experiment repeated twice.

Fig. 5. Detection of apoptosis by the TdT assay in CML CD34⁺ cells exposed to AA. Cells were incubated for 18 h without or with AA (100 μM). Percentages of apoptotic cells in the live gated cell population are indicated. A, vehicle-treated cells (Control); B, AA-treated cells.

Fig. 6. Detection of DNA fragmentation by gel electrophoresis in CML CD34⁺ cells exposed to AA. Cells (1 × 10⁶/lane) were treated with vehicle (Lane 2) or with AA (100 μM for 18 h; Lane 3). DNA was extracted and resolved by 2% agarose gel electrophoresis. Lane 1, DNA (2.1–0.15 kbp) marker standards. Arrowheads, low molecular weight DNA fragments.
more, NDGA and ketoconazole, in the absence of AA, induced a modest inhibition of cell growth (data not shown). Thus, it may be possible that lipooxygenase or cytochrome P450 metabolites are involved in supporting proliferation of CML cells. Nevertheless, the results presented here strongly suggest that AA itself is implicated in inhibition of growth and apoptosis of H7.bcr-abl A54 cells.

Among the other fatty acids tested, only γ-linolenic acid inhibited H7.bcr-abl A54 cell proliferation to a greater extent than did equimolar concentrations of AA. The antiproliferative effect of γ-linolenic acid is likely related to its further conversion into AA, although it is possible that γ-linolenic acid exerts its effect directly (43).

The possibility of lipid peroxidation pathways acting in the signaling route of AA-induced inhibition of cell growth was also investigated. Several investigations have shown that lipid peroxides derived from polyunsaturated fatty acids inhibit proliferation of a variety of cell types and that addition of antioxidants to the culture medium blocked the inhibitory effect of fatty acids (51). Moreover, intracellular oxidants, including free radicals or oxidized intermediates of lipids, are closely involved in induction of apoptosis (52). Here, we observed that inhibition of H7.bcr-abl A54 cell proliferation by AA was not blocked by coinubcation with antioxidants, suggesting that reactive oxygen species do not mediate the effect of AA on cell growth. Among the antioxidants tested, the iron chelator desferrioxamine caused inhibition of cell growth in the absence of AA. This effect is likely due to the requirement of iron for cell growth (53). However, in the presence of desferrioxamine, inhibition of growth by AA was further enhanced, suggesting an additive effect of AA and desferrioxamine on cell proliferation. Further studies are required to investigate this possibility.

In conclusion, the results presented here demonstrated a role for AA in the induction of apoptosis in CML. The signal transduction pathway of AA-induced inhibition of cell growth did not involve reactive oxygen radicals or arachidonate metabolites. Finally, primary CML cells and bcr-abl transformed cell lines were more sensitive to the antiproliferative effect of AA than were their normal counterparts. Further investigations are warranted to identify immediate targets involved in conferring a greater susceptibility of bcr-abl cells to the antiproliferative effect of AA. Identification of these specific targets may have profound therapeutic implications.

Fig. 7. Effect of fatty acids on proliferation of H7.bcr-abl A54 cell line. Cells (1 × 10^5/ml) were stimulated in media containing 10% FBS with various fatty acids at 50 μM, including AA, palmitic acid (PA), oleic acid (OA), linoleic acid (LA), α-linolenic acid (LnA), and γ-linolenic acid (γ-LnA) for 72 h. Con, control. MTS assay was performed as described in “Materials and Methods.” Columns, mean absorbance units from a representative of three experiments; bars, SD.

Fig. 8. Effect of inhibitors of AA metabolism on arachidonate-induced inhibition of cell growth. H7.bcr-abl cells (1 × 10^5/ml) were incubated for 30 min with indomethacin (10 μM), caffeic acid (10 μM), NDGA (10 μM), or ketoconazole (10 μM) prior to stimulation with AA (50 μM) for 72 h. Cell proliferation was determined by the MTS assay. Columns, mean absorbance units from a representative experiment performed in triplicate; bars, SD.

Fig. 9. Effect of ETYA on H7bcr.abl A54 cell proliferation. Cells (1 × 10^5/ml) in 24-well plates were stimulated with the indicated concentrations of ETYA. Cell proliferation was determined at 72 h by the MTS assay. Columns, mean absorbance units from a representative experiment performed in triplicate; bars, SD.

Fig. 10. Effect of antioxidants on AA-induced inhibition of cell growth. H7.bcr-abl cells (1 × 10^5/ml) were incubated for 30 min with vitamin E (Vit. E, 200 μg/ml; A), catalase (Cat., 1000 units/ml; B), or desferrioxamine (DSF, 0.3 mM; C), prior to stimulation with AA (50 μM) for 72 h. Cell proliferation was determined by the MTS assay. Columns, mean absorbance units from a representative experiment performed in triplicate; bars, SD.
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