Induction of Apoptosis and Cell Cycle Arrest in Mouse Colon 26 Cells by Benastatin A

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Benastatin A, isolated from Streptomyces bacteria, is reported to inhibit mammalian glutathione transferases (GSTs). Since GST inhibitors such as ethacrynic acid are suggested to induce apoptosis in some cell lines, the effect of benastatin A on the survival of mouse colon 26 adenocarcinoma cells was compared with that of ethacrynic acid. When cells in stationary phase were treated with benastatin A, viable cells were found to be dose-dependently decreased after 3 days. In the case of ethacrynic acid, this became apparent within 24 h. Electrophoretic analysis revealed DNA fragmentation, indicating that cell loss was due to apoptosis in both cases. The dominant GST in colon 26 cells was identified as the class Pi-form (GST-II), and the activities in crude extracts as well as purified GST-II were almost completely inhibited by 50 μM ethacrynic acid. Immunoblot and northern blot analyses revealed increased GST-II protein and mRNA levels in cells treated with ethacrynic acid. Benastatin A did not significantly affect the activity in the crude extract even at 20 μM, a 10-fold higher concentration than that which almost completely inhibited the activity of purified GST-II. However, GST activity and GST-II protein were decreased in colon 26 cells treated with benastatin A for 5 days, no significant activity being detected in the range of 16–20 μM. In addition, ß-actin and bax mRNAs were also decreased in a dose-dependent manner. Furthermore, flow cytometric analysis of colon 26 cells revealed that benastatin A blocked the cell cycle at the G1/G0 phase. Thus, benastatin A also induces apoptosis of colon 26 cells, but this is unlikely to be due to inhibition of GST activity.

Key words: Glutathione transferase — Enzyme inhibitor — Apoptosis — Cell cycle — Colon cancer

GSTs (EC 2.5.1.18) are a family of dimeric enzymes which perform multiple functions, including conjugation of GSH with a number of electrophilic compounds.1) Mammalian cytosolic GSTs have been grouped into five classes, α, μ, π, θ, and σ, on the basis of enzymatic and immunological properties, and similarities in nucleotide and amino acid sequences.2) π-class GSTs, such as rat GST-P (7-7) and mouse GST-II, are especially interesting because their expression has been associated with neoplastic development and drug resistance.3, 4) π-class GSTs are increased in many cancer cell lines resistant to alkylating agents, doxorubicin and cis-diaminedichloroplatinum (II), as compared with respective sensitive cell lines.5) Transfection studies with cDNAs encoding GSTs6) and studies utilizing inhibitors for GST7) have supported an involvement of the enzyme in drug resistance.

Apoptosis was first described as a morphologic pattern of cell death characterized by shrinkage and chromatin condensation, resulting in cell fragmentation.8) One hallmark of the process is internucleosomal DNA cleavage.9) The p53 protein is linked to either apoptosis or cell cycle arrest, depending on cell types.10–12) Other genes such as bcl-2 are associated with inhibition of cell death13) while the Bax protein, related to the Bcl-2 in its primary structure, induces apoptosis.14) JNK, a member of the mitogen-activated protein kinase family, transduces information from the extracellular environment, such as cytokine signals and oxidative stress, into the nucleus, resulting in a stress response15) and apoptosis induction.16) Apoptosis is known to occur in a variety of physiological and pathological situations. Recent studies have revealed that many anti-cancer drugs such as cis-diaminedichloroplatinum (II) and etoposide induce apoptotic features in cancer cells, but that this does not occur in cell lines resistant to the drugs,17) so that the outcome of chemotherapy may be determined by the inducibility of apoptosis.18)
GST inhibitors such as EA have been demonstrated to induce apoptosis in the Jurkat human T cell line\(^{29}\) and other cell lines,\(^{20}\) suggesting that GSTs expressed in resistant cancer cells play roles of detoxification of anti-cancer drugs and prevention of apoptosis. BA (8,13-dihydro-1, 7, 9, 11-tetrahydroxy-13-dimethyl-8-oxo-3-pentyl-benz[a]naphthacene-2-carboxylic acid) isolated from the culture broth of \textit{Streptomyces} sp. MI384-DF12 has been shown to inhibit rat GSTs independent of their class.\(^{21, 22}\) However, there have been no reports on apoptosis induction by benastatin.

In the present study, the effect of BA on the survival of mouse colon 26 cells expressing large amounts of GST-II\(^{23}\) was compared with that of EA. BA also induced apoptosis, but did not inhibit GST activity in colon 26 extracts, indicating that the underlying mechanisms differ between the two drugs.

**MATERIALS AND METHODS**

**Materials** BA was prepared as described previously.\(^{21}\) CDNB, and 4-chloro-1-naphthol were purchased from Wako Pure Chemical (Osaka). EA, actinomycin D, and cycloheximide were from Sigma (St. Louis, MO) and GSH was from Kojuni Co., Ltd (Tokyo). Alamar Blue was from Alamar Biosciences Inc. (Sacramento, CA). Inhibitors of caspase-1 and caspase-3, tetrapeptide aldehydes Ac-YV AD-CHO and Ac-DEVD-CHO, respectively,\(^{24}\) were from the Peptide Institute (Osaka). A random-primed DNA labeling kit was purchased from Amersham (Buckinghamshire, UK) and [\(\alpha\)-32P\]dCTP from NEN (Boston, MA). Nitrocellulose papers were from Schleicher and Schuell (Keene, NH), and anti-rabbit IgG horseradish peroxidase antibodies were generous gifts from Dr. H. Ariga (Hokkaido University School of Medicine, Sapporo) and Dr. Y. Tsujimoto (Osaka University School of Medicine, Suita), respectively. cDNAs for human GAPDH and mouse \(\beta\)-actin were generous gifts from Dr. H. Ariga (Hokkaido University School of Pharmacy, Sapporo). Mouse \textit{bax} cDNA was kindly provided by Dr. S. J. Korsmeyer (Washington University School of Medicine, St. Louis, MO)\(^{14}\) and rat \textit{IL6-DBP} (or silencer factor-B) cDNA by Dr. M. Imagawa (Osaka University School of Pharmacy, Suita).\(^{27}\)

**Cell culture and measurement of cell number** Colon 26 and Ehrlich ascites cells were supplied by the Japanese Cancer Research Resources Bank (JCRB, Tokyo). Colon 26 cells were grown in RPMI1640 medium (Nissui Pharmaceutical Co., Tokyo), containing 10% (v/v) heat-inactivated FCS (JRH Biosciences, Lenexa, KS). Ehrlich ascites cells were maintained in NCTC135 medium (Gibco, Paisley, UK) supplemented with 10% FCS. All cultures were performed at 37°C under 5% CO\(_2\) in air. Cell number was determined according to the method of Owens and Belcher.\(^{33}\) Viable cells were also counted on the basis of trypan blue exclusion.\(^{29}\)

**Analysis of DNA fragmentation** Total DNAs were extracted from 2\(\times\)10\(^6\) cells following the method of Sambrook \textit{et al.}\(^{30}\) and 5 \(\mu\)g aliquots were separated by 1.4% (w/v) agarose gel electrophoresis in 40 mM Tris-5 mM sodium acetate-1 mM EDTA (pH 7.8) and stained with ethidium bromide. DNA bands were visualized under UV light.

**Assay for GST activity** Cells were disrupted in a solution of 10 mM Tris-HCl, pH 7.8, containing 0.2 \(M\) NaCl by sonication for 30 s 4 times using a sonifier (model UR-20P, Tomy Seiko Co., Tokyo), and centrifuged at 105 000 \(g\) for 30 min. The supernatants were used for assay of GST activity according to the method of Habig \textit{et al.},\(^{31}\) employing CDNB as the substrate. Protein amounts were determined by the method of Bradford.\(^{32}\) Total glutathione, reduced and oxidized glutathione, was determined according to the method of Owens and Belcher.\(^{33}\) The activity inhibition test by anti-GST-II antibody was performed as reported previously.\(^{34}\) Enzyme preparation and anti-GST-II antibody mixtures were incubated at 4°C overnight and centrifuged at 16 000 \(g\) for 10 min, then the supernatants were assayed for GST activity.

**SDS-PAGE and immunoblotting** SDS-PAGE was performed with 12.5% acrylamide gels by the method of Laemmli.\(^{35}\) Proteins were stained with Coomassie Brilliant Blue R-250. For immunoblotting, they were transferred to nitrocellulose filters, and visualized with anti-GST-II antibody, raised in a rabbit as reported previously,\(^{36}\) according to the method of Towbin \textit{et al.}\(^{37}\)

**RNA preparations and northern blotting** RNAs were prepared from colon 26 cells by a standard method.\(^{30}\) Twenty microgram samples denatured with formaldehyde and formamide were separated on 1% (w/v) agarose gels containing 1.1 \(M\) formaldehyde and transferred to nylon membranes (Hybond-N, Amersham) in 20\(\times\) SSC (3 \(M\) NaCl-0.3 \(M\) Na citrate, pH 7.0). All cDNA probes, includ-
ing mouse GST-II cDNA (pGM211), were labeled by the random priming method. Hybridization was performed with 3P-labeled cDNA probes at 42°C for 18 h in 50% formamide-3× SSC-0.05 M Tris-HCl (pH 7.5)-1 mM EDTA-1× Denhardt-20 μg/ml tRNA-20 μg/ml herring sperm DNA. Then the filters were washed twice with 3× SSC-0.1% SDS at 37°C for 30 min, and twice with 0.1× SSC-0.1% SDS at 50–65°C for 30 min. Autoradiography was carried out by exposure of X-ray films (Kodak X-Omat AR, Eastman Kodak, Rochester, NY) at −80°C using an intensifying screen. Results of autoradiography were quantified with a densitometer (Model-Pan, Jookoo Co., Tokyo) and the values were expressed relative to the GAPDH mRNA level.

Flow cytometry and cell cycle analysis Exponentially proliferating colon 26 cells, suspended at a density of 1×10⁵ cells per well of 6-well flat-bottomed plates (corresponding to 3000 cells per well of a 96-well plate) and cultured for 24 h, were treated with 16 μM BA for 3 days. To synchronize cells at the G2/M boundary, nocodazole, an inhibitor of microtubule polymerization, was added to the culture medium at a final concentration of 0.13 μM, 24 h before harvest. Cells were then fixed in 70% ethanol and, after treatment with RNase (Amresco, Solon, OH), were stained with 50 μg/ml of PI (Molecular Probes Inc., Eugene, OR). Fluorescence was detected with a flow cytometer (FACScan, Becton Dickinson Co., San Jose, CA) and the CellFit software system was employed to analyze cell cycle parameters.

RESULTS

Effects of BA and EA on the viability of colon 26 cells To study the effects of BA and EA on the viability of colon 26 cells, viable cell number was estimated by the Alamar Blue assay and expressed as intensity of fluorescence. When colon 26 cells in the stationary phase (10 000 cells/well) were treated with 12–20 μM BA, fluorescence was dose-dependently decreased on days 4 and 5, as compared with the initial value (Fig. 1A). The dye exclusion test confirmed the decrease in viable cells. With EA, reduction in fluorescence was already dose-dependent at day 1 and then remained relatively constant (Fig. 1B). When colon 26 cells in the exponentially growing phase (plated at 300 cells/well) were treated with BA, cell proliferation was dose-dependently inhibited, but no decrease in viable cells was evident (data not shown). After the removal of BA, colon 26 cells exhibited a proliferation rate similar to that of control cells, indicating the reversible nature of the inhibition. On the other hand, when Ehrlich ascites cells were incubated with BA (Fig. 1C), such decreases were not observed.

On microscopic examination, 10 000 cells/well of colon 26 incubated with 20 μM BA for 3 days showed changes in cell shape accompanied with aggregation of nuclei and cytoplasmic loss (Fig. 2). When colon 26 cells were exposed to BA for 2 days and cultured for a further 3 days in medium without the drug, cells retained a normal morphology. Morphological alterations also occurred within 2 days in colon 26 cells treated with 60–80 μM EA (data not shown).

Induction of apoptosis of colon 26 cells by BA and EA To examine whether the decrease of cell numbers by BA or EA (Fig. 1, A and B) was due to apoptosis, DNA fragmentation was analyzed by electrophoresis. DNA ladder formation was observed in colon 26 cells treated with 12–20 μM BA for 5 days (Fig. 3A). At a 20 μM concentration, such ladders were not evident for the initial 2 days but were apparent at day 3 and thereafter (Fig. 3B). Incubation of colon 26 cells with 50–80 mM EA for 2 days as
well as for 5 days also resulted in DNA fragmentation (Fig. 3C). DNA fragmentation induced by these drugs was not blocked by the addition of inhibitors of caspase-1 or caspase-3 (data not shown). DNA ladders were also observed for colon 26 cells treated with actinomycin D or cycloheximide, and the time course of DNA fragmentation was similar to that induced by BA. No DNA fragmentation was observed with Ehrlich ascites cells treated with BA or EA (data not shown).

**Effects of BA and EA treatments on GST and other mRNA levels in colon 26 cells** We examined whether the addition of BA and EA to crude extracts of colon 26 cells could inhibit the GST activity. Although the activity of purified GST-II was almost completely inhibited by 2–4 µM BA, that in crude extracts was hardly affected up to 10 µM and only 30% inhibited at 20 µM (Fig. 4A). Unlike the BA case, similar dose-dependent inhibition patterns for crude extract and purified GST-II preparations were seen with EA (Fig. 4B). The activity inhibition patterns of both crude extract and purified GST-II preparations by anti-GST-II antibody were essentially identical and the remaining activity of crude extract approached zero with increase in the amount of the antibody (data not shown), indicating the dominant GST form in colon 26 cells to be GST-II.

Fig. 2. Morphological changes in colon 26 cells due to BA. (A) Photograph of colon 26 cells treated with 20 µM BA for 3 days. (B) Control colon 26 cells without BA. Original magnification, ×100.

Fig. 3. DNA fragmentation in colon 26 cells due to BA or EA. Cells of colon 26 (2×10⁶) were incubated in the presence of various concentrations of BA (A, 0–20 µM) or EA (C, 0–80 µM) for 5 days. Total DNAs were then extracted and analyzed by 1.4% agarose gel electrophoresis. (B) Time course of DNA fragmentation in colon 26 cells treated with 20 µM BA for 1–5 days. M, DNA size markers (λ-HindIII digest + φx174-HaeIII digest).

Fig. 4. Inhibition of GST activities of crude colon 26 extract and purified GST-II due to BA or EA in vitro. Crude extracts of colon 26 cells (○) or purified GST-II (●) were incubated with 0–20 µM BA (A) or 0–100 µM EA (B) and then assayed for GST activity with CDNB. The activity in the presence of the inhibitor was expressed as the percentage of that without the inhibitor. Each point and bar represent the mean and SD from triplicate assays.
culture medium. GST activity expressed per 10^6 live cells was dose-dependently decreased following BA treatment, no significant activity being detected in the range of 16–20 μM (Fig. 5A). GST activity expressed per protein amount was also decreased in a dose-dependent manner (data not shown). In contrast, GST activities of colon 26 cells treated with 50–70 μM EA were increased up to 2-fold, as compared with the control (Fig. 5B). Immunoblotting with anti-GST-II antibody revealed a marked decrease of GST-II in cells treated with 16–20 μM BA (Fig. 5C, lanes 4 and 5), while, consistent with GST activity, treatment with 50–70 μM EA resulted in an increase (lanes 7–9).

The effects of 2-day BA or EA treatments on the levels of GST-II mRNA and other mRNAs in colon 26 cells were examined. The results of northern hybridization were quantitated by densitometry and the values were expressed relative to the GAPDH mRNA level. Since treatment with 20 μM BA for 2 days resulted in degradation of rRNA (Fig. 6A), most analyses were performed at up to 16 μM. GST-II mRNA levels were hardly affected by up to 12 μM BA but decreased to about 75% of the control value at 16 μM (Fig. 6B). The levels of β-actin, IL6-DBP, bax and c-jun mRNAs were all decreased in a dose-dependent manner in the range of 8–16 μM, although the extent varied with the individual mRNA. EA at 50–70 μM significantly increased GST-II mRNA levels (Fig. 6, A and C), consistent with increased GST-II protein levels (Fig. 5, B and C). Although β-actin and IL6-DBP mRNA levels were not altered in this case, the bax mRNA level was increased, while c-jun mRNA decreased.

**Cell cycle analysis of colon 26 cells treated with BA** In order to determine whether BA altered cell cycle progres-

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**Fig. 5.** Effects of BA and EA on GST activity and GST-II protein levels in colon 26 cells. Aliquots of 2×10^6 colon 26 cells were incubated with BA (0–20 μM, A) or EA (0–80 μM, B) for 5 days. Then, total proteins were extracted and activities towards CDNB were measured and expressed per 10^6 live cells. Each point and bar represent the mean and SD from triplicate assays. (C) The cell extracts as described above were analyzed for GST-II by immunoblotting with anti-GST-II antibody. Lanes 1, 2, 3, and 5 contain extracts derived from colon 26 cells treated with 0, 8, 12, 16, and 20 μM BA, respectively, and lanes 6, 7, 8, 9, and 10 those for 0, 50, 60, 70, and 80 μM EA. Each lane contains 50 μg of protein.

**Fig. 6.** Effects of BA and EA on GST-II and other mRNA levels in colon 26 cells. (A) Total RNAs were extracted from colon 26 cells treated with various concentrations of BA (0–20 μM) or EA (0–80 μM) for 2 days and the levels of GST-II and other mRNAs (c-jun, bax, and GAPDH) were analyzed by northern hybridization. Each lane contains 20 μg of total RNAs and the RNA staining pattern is shown at the bottom. (B and C) Autoradiograms were quantified by densitometry and the results expressed relative to the GAPDH mRNA level. , GST-II mRNA; ○, c-jun mRNA; △, bax mRNA; ▼, β-actin mRNA; □, IL6-DBP mRNA. (B) mRNA levels in colon 26 cells treated with 0–20 μM BA. (C) Results for treatment with 0–80 μM EA.
DISCUSSION

The present study revealed that two GST inhibitors, BA and EA, induce apoptosis in colon 26 cells in the stationary phase, but have very different effects on the various parameters investigated. BA down-regulated not only GST-II mRNA production, but also that of other mRNAs in colon 26 cells, β-actin, bax, and c-jun mRNAs all being affected. It was unlikely that these decreases reflected loss of mRNAs by cell death, because they were not observed in common in cells treated with EA exhibiting a similar degree of apoptosis. Furthermore, BA also induced cell cycle arrest at the G0/G1 phase. These effects exerted by BA are unlikely to be due to inhibition of GST activity. Although BA inhibited the activity of purified enzyme, it did not inhibit that in crude extracts of colon 26 cells. However, since apoptosis induction and inhibition of cell proliferation by BA was not detected in Ehrlich ascites cells, its effects seemed to be limited to certain cell lines.

Both actinomycin D and cycloheximide were found to induce apoptosis in colon 26 cells, as reported earlier for human HL-60 cells, although these drugs are known to block p53-mediated apoptosis in rat fibroblasts. The time course of apoptosis induction following BA treatment was similar to that induced by actinomycin D or cycloheximide. These findings raise the possibility that apoptosis by BA is dependent on repression of survival factors rather than synthesis of apoptosis-inducing proteins, although the exact targets of BA remain to be identified. The effects exerted by BA were diverse: induction of apoptosis, repression of transcription, and cell cycle arrest. Thus, targets of BA may be involved in these processes of biological importance. p53 and members of the general transcription factor complexes such as TFIIF are known to modulate apoptosis and the cell cycle. Further studies are clearly needed to examine whether BA indeed inhibits the function of these proteins.

EA enhanced GST-II protein and mRNA levels, but inhibited the GST activity of crude extracts as well as that of the purified enzyme. Such induction of GST has been reported in a few cell lines after treatment with EA or other GST inhibitors. Apoptosis was induced within a day in EA-treated cells. DNA fragmentation bands were more prominent at 50–60 μM, as compared with those at 70–80 μM (Fig. 3C). In this experiment, EA treatment was continued for 5 days. As shown in Fig. 1B, viable cells decreased on day 1 upon treatment with 60–80 μM EA, and most cells had been lost by day 5. At 50–60 μM EA, the decrease in viable cells was minimal and apoptosis was shown to proceed on day 5, suggesting that both apoptosis and cell proliferation proceeded simultaneously. Since inhibition of GST by EA and other drugs is reported to induce apoptosis in some cell lines, apoptosis by EA in the present study may be dependent on inhibition of GST activity. Since GST has been demonstrated to form complexes with JNK, a member of the mitogen-activated protein kinase family, and to inhibit the kinase activity, this could regulate stress responses and apoptosis induction. Several GST inhibitors have been demonstrated to release JNK from GST complexes, and then free JNK can phosphorylate and activate c-Jun. Since GST-II expression is suggested to be under the control of c-Jun, such
activation of c-Jun may be involved in enhanced GST-II expression following EA treatment. The Bax protein induces apoptosis in lymphoid cells possibly by blocking the survival function of Bcl-2 protein through the formation of heterodimers.14) Bax or Bcl-2-mediated apoptosis is reported to be prevented by inhibitors of caspase-1 and caspase-3.24,42) The observed increase in bax mRNA in EA-treated cells is unlikely to be directly responsible for apoptosis induction, since the caspase inhibitors as described above did not prevent it (data not shown).

In conclusion, BA induces apoptosis and cell cycle arrest in colon 26 cells and also represses transcription. These effects are unlikely to be due to inhibition of GST activity. Thus, the underlying mechanisms of apoptosis induction differ between BA and EA.

ACKNOWLEDGMENTS

This work was supported in part by research grants from the Karoji Memorial Fund of Hirosaki University School of Medicine and from the Ichiro Kanehara Foundation.

(Received May 10, 2000/Revised August 2, 2000/Accepted August 12, 2000)

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