Involvement of Hydrogen Peroxide Production in Erbstatin-induced Apoptosis in Human Small Cell Lung Carcinoma Cells

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ABSTRACT

Tyrosine kinase inhibitor, erbstatin, induced morphological apoptosis and DNA fragmentation in human small cell lung carcinoma (SCLC) cells. Erbstatin-induced apoptosis was inhibited by antioxidants, whereas erbstatin-inhibited tyrosine phosphorylation was not affected by them. Erbstatin was shown by means of flow cytometry to induce hydrogen peroxide generation. Furthermore, hydrogen peroxide induced morphological apoptosis and DNA fragmentation in the SCLC cells. We also demonstrated that erbstatin-induced hydrogen peroxide production and DNA fragmentation were partially suppressed by inhibition of protein synthesis. Thus, erbstatin-induced apoptosis would be due to hydrogen peroxide generation via newly synthesized protein.

INTRODUCTION

Apoptosis can be induced by alteration of various cellular signaling pathways. It has been demonstrated that an increase in the cytosolic calcium level induced cell activation and apoptosis in lymphocytes (1). Protein kinase C has been shown to be a critical molecule for both calcium level-induced cell activation and apoptosis (2). It is unclear at which point the same signal is altered, leading to proliferation or apoptosis. Therefore, crucial mediators that determine these very different biological consequences are considered to lie hidden. Recently, protein tyrosine kinase activity was reported to regulate the process of apoptosis. Abi protein, a nonreceptor type tyrosine kinase, inhibited cytokine withdrawal-mediated (4) or Fas-mediated apoptosis (5). Inhibition of a number of the Janus family of protein tyrosine kinases (Jak2) induced apoptosis in acute lymphoblastic leukemia (6). Protein tyrosine kinase activity thus seems to be necessary for the prevention of apoptosis. Indeed, we and others demonstrated that inhibition of protein tyrosine kinase by its inhibitors, such as erbstatin, herbimycin A, or genistein, induced apoptosis in lymphocytes (7, 8). However, the intracellular events that lead to apoptosis following tyrosine kinase inhibition are still obscure.

Emerging evidence has indicated the involvement of ROS,3 including singlet oxygen, superoxide, hydrogen peroxide, hydroxyl radical, and nitric oxide, in the signaling pathway for apoptosis. Overexpression of Bcl-2, an inhibitor of apoptosis, decreased ROS generation and increased resistance to apoptotic killing by hydrogen peroxide, menadione, and depletion of glutathione (9). On the other hand, the expression level of Bcl-2 in cytokine-dependent cells was suppressed by treatment with herbimycin A (10). Therefore, we decided to examine involvement of ROS in a protein tyrosine kinase-dependent pathway leading to apoptosis.

In the present study, we demonstrated that erbstatin, a protein tyrosine kinase inhibitor, induced apoptosis without affecting the expression level of Bcl-2 in human SCLC cells and obtained data indicating that erbstatin-induced apoptosis may be due to hydrogen peroxide production via newly synthesized protein.

MATERIALS AND METHODS

Materials. Erbstatin and herbimycin A were isolated from Streptomyces as described previously (11, 12). Hoechst 33258 and DCFH-DA were obtained from Polysciences (Warrington, PA) and Molecular Probes (Eugene, OR), respectively. [3H]Thymidine (45.5 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, IL).

Cell Culture. Human SCLC cell lines Ms-1 and Ms-13 and a non-SCLC cell line Ma-44 were cultured in RPMI 1640 containing 10% fetal bovine serum, penicillin G (100 units/ml), and kanamycin (0.1 mg/ml) at 37°C in a 5% CO2-95% air atmosphere.

Hoechst 33258 Staining. Hoechst 33258 staining was performed as described before (13). In brief, cells were fixed with 3% paraformaldehyde for 20 min at room temperature, washed with distilled water, and dried. Then, the cells were stained with Hoechst 33258 (10 µg/ml) for 5 min, washed, and examined by fluorescence microscopy. Viable and apoptotic cells revealed round nuclei and fragmented nuclei, respectively (14, 15).

Analysis of DNA Fragmentation. Ms-1 cells were washed and lysed in lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 0.5% sodium N-lauroyl sarcosinate, and 100 µg/ml proteinase K, pH 8.0) for 5 h at 4°C. The lysates were extracted with phenol and chloroform, and the DNA in the aqueous layer was precipitated with ethanol following the addition of sodium acetate (final concentration, 0.3 M). The DNA was then collected by centrifugation and dried. The samples were finally dissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), incubated at 37°C for 3 h with RNase A (1 µg/ml), and electrophoresed on a 1.5% agarose gel.

Western Blotting. The cells were lysed in a lysis buffer (25 mM HEPES, 1.5% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.5 mM Na3EDTA, 50 mM NaF, 0.1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mg/ml leupeptin, pH 7.8) at 4°C with sonication. The resulting compound, dichlorofluorescin (DCFH), is reactive with hydrogen peroxide to give a fluorescent compound, dichlorofluorescin diacetate. DCFH-DA can permeate cell membranes and then be deacetylated by intracellular esterases. The resulting compound, dichlorofluorescin (DCF), was performed with enhanced chemiluminescence reagent (DuPont, Boston, MA).

Evaluation of Intracellular Hydrogen Peroxide. The cells were treated with chemicals in the presence of 5 µM DCFH-DA. The esterified form of DCFH-DA can permeate cell membranes and then be deacetylated by intracellular esterases. The resulting compound, dichlorofluorescin (DCF), is reactive with hydrogen peroxide to give a fluorescent compound, dichlorofluorescein (DCF) (16). The amount of intracellular hydrogen peroxide was detected by flow cytometry (Epics Elite; Coulter, Hialeah, FL).

Analysis of SOD and Catalase Activities. After treatment with erbstatin, Ms-1 cells were washed, suspended in 100 µl of H2O2, and disrupted by four cycles of freezing at -70°C and thawing at 60°C. The lysis was centrifuged at 14,000 rpm for 15 min at 4°C, and the supernatant was electrophoresed on the polyacrylamide gel under nondenaturing conditions. The SOD activity was determined by reduction of nitroblue tetrazolium. SOD activity can be observed as a transparent band in the blue formazan pigment background (17). Catalase activity was detected with diaminobenzidine. This method is con-
ceived as a negative stain, in the sense that a band of catalase in the gel decompose hydrogen peroxide and thus prevent the chromogenic peroxidase-catalyzed oxidation of the diaminobenzidine, yielding an achromatic band against a uniformly stained background (18).

**Measurement of DNA Fragmentation Ratio.** DNA fragmentation was assayed by the method described by Kamesaki et al. (19) with slight modifications. The cells were labeled with [³H]thymidine (0.5 μCi/ml) in fetal bovine serum-free RPMI for 2 h. Then, the cells were washed and suspended in medium with or without agents. After treatment with test chemicals, the cells were collected and lysed with lysis buffer (5 mM Tris-HCl, 20 mM EDTA, and 0.5% Triton X-100, pH 8) for 10 min on ice. The lysates were centrifuged at 15,000 X g for 30 min, and the radioactivities in the pellet and supernatant were counted in a liquid scintillation counter. The supernatant count was taken as fragmented DNA. The supernatant count of the untreated cells was subtracted from all of the results.

**RESULTS**

**Induction by Erbstatin of Apoptosis in SCLC Cells.** Erbstatin gradually decreased the viability of Ms-l cells, as determined by trypan blue dye exclusion. As shown in Fig. 1A, this effect of erbstatin was dose and time dependent. After 16 h in culture, about 80% of the cells were killed by 30 μg/ml erbstatin (167 μM); and at this time point, nuclear fragmentation and condensation were observed in the cells (Fig. 1B). Similar results were obtained in SCLC Ms-13 and non-SCLC Ma-44 cells. Furthermore, erbstatin dose-dependently induced DNA fragmentation in the Ms-1 cells (Fig. 1C). These results indicate that erbstatin induced apoptotic cell death in Ms-1 cells.

**Effect of Antioxidants on Erbstatin-induced Death of Ms-1 Cells.** Next, we tested the effect of antioxidants on erbstatin-induced cell death. NAC (2 mM), GSH (2 mM), and DTT (0.5 mM), which are known to scavenge H₂O₂, abolished erbstatin-induced cell death, as shown in Fig. 2. NAC and GSH also inhibited erbstatin-induced cell death in Ms-13 cells and Ma-44 cells. N-Acetylserine, a compound lacking the scavenging function of H₂O₂, did not affect erbstatin-induced cell death, even at a concentration of 10 mM (20). Moreover, neither a scavenger of lipid peroxidation, α-tocopherol (< 1 mM; Ref. 21) nor an inhibitor of nitric oxide synthetase, N°-monomethyl-L-arginine (0.1—5 mM; Ref. 22), abrogated cell death induced with erbstatin (data not shown). Next, we examined whether suppression of erbstatin-induced cell death by NAC was due to the inactivation of erbstatin itself by NAC. As shown in Fig. 3, erbstatin (30 μg/ml)
induced tyrosine phosphorylation of 130–150-kDa proteins in a time-dependent manner in Ms-1 cells. In the presence of 2 mM NAC, the inhibitory activity of erbstatin was not changed (Fig. 3). Additionally, NAC did not affect the inhibition by erbstatin of tyrosine kinase in vitro (data not shown). Therefore, these results suggest that prevention of erbstatin-induced apoptosis by antioxidants was not due to the inactivation of erbstatin. Herbimycin A-induced cell death was again inhibited by NAC (data not shown); however, herbimycin A itself is known to be inactivated by sulfhydryl compounds, resulting in the elimination of its inhibitory activity toward tyrosine kinase (23).

**Induction of Hydrogen Peroxide Generation by Erbstatin in Ms-1 Cells.** To determine whether H2O2 was actually generated by the treatment of Ms-1 cells with erbstatin, we used DCFH-DA to detect the amount of intracellular H2O2. The content of H2O2 in erbstatin (30 μg/ml)-treated Ms-1 cells was gradually increased, as shown in Fig. 4A. These effects were also observed in Ms-13 cells and Ma-44 cells. Herbimycin A also induced H2O2 production (data not shown). Furthermore, the increase in H2O2, caused by erbstatin, was eliminated by the addition of NAC or GSH (Fig. 4A). Taken together, these data suggest that inhibition of tyrosine kinase would increase the intracellular H2O2 concentration, thereby inducing apoptotic cell death in Ms-1 cells.

The amount of intracellular H2O2 may be regulated by SOD and catalase. Therefore, we examined the effect of erbstatin on SOD and catalase activities. However, neither enzyme activity was affected by the treatment with 30 μg/ml erbstatin up to 4 h, as shown in Fig. 4, B and C, respectively. We next analyzed the Bcl-2 expression level in erbstatin-treated cells. As shown in Fig. 4D, the protein level of Bcl-2 was not decreased by erbstatin (30 μg/ml) up to 4 h. Herbimycin A also failed to reduce the expression level of Bcl-2 in Ms-1 cells (data not shown).

**Involvement of Newly Synthesized Protein in Erbstatin-induced Apoptosis in Ms-1 Cells.** Because inhibitors of RNA or protein synthesis suppress several types of apoptosis (24), we examined the effect of these inhibitors on erbstatin-induced apoptosis. By the treatment of Ms-1 cells with erbstatin for 16 h, about 30% DNA fragmentation was observed in the cells, as determined by the release of fragmented DNA from [3H]thymidine-labeled cells (Fig. 5A). However, when the cells were pretreated with 10 μg/ml of CHx for 1 h, under conditions in which more than 80% of the protein synthesis was inhibited (data not shown), there was a partial but significant decrease in the DNA fragmentation ratio at 16 h following erbstatin addition to the Ms-1 cells (Fig. 5A). Thus, erbstatin-induced DNA fragmentation would require new protein synthesis. On the other hand, H2O2-induced DNA fragmentation was not inhibited by CHx (Fig. 5F). Therefore, we examined whether the increase in H2O2 level in erbstatin-treated cells was suppressed by CHx. As shown in Fig. 6, pretreatment of cells with CHx abolished the increase in H2O2 concentration induced with erbstatin. Similar results were obtained by pretreatment with 1 μg/ml of actinomycin D. The herbimycin A-induced increase in H2O2 was also eliminated by CHx as well as by actinomycin D. These results suggest that new protein induced by erbstatin is involved in the generation of a high amount of intracellular H2O2, leading to apoptosis.

**DISCUSSION**

Erbstatin was originally isolated from *Streptomyces* as an inhibitor of epidermal growth factor-receptor tyrosine kinase (11). It also inhibits nonreceptor-type tyrosine kinases, such as Src, Fyn, and Lck, whereas it does not inhibit serine/threonine protein kinases, such as protein kinases C and protein kinase A. 5′-O-Methylerbstatin, an inactive analogue of erbstatin, does not induce apoptosis (data not shown). Furthermore, herbimycin A, another tyrosine kinase inhibitor, also induces apoptotic cell death in SCLC cells. Therefore, tyrosine kinase is likely to be involved in the pathway preventing apoptosis in SCLC cells.

Recent data have indicated that ROS may play a crucial role in the mechanisms underlying cellular death. For example, some antioxidants often inhibit apoptosis (25). Also, we found in the present study that erbstatin-induced cell death in SCLC cells was diminished by antioxidants. NAC, GSH, or DTT are all known to be H2O2 scavengers. Therefore, all of them would seem to suppress erbstatin-induced apoptosis by acting as an H2O2 scavenger. On the other hand, lipid radicals and nitric oxide (26) are probably not involved, since α-tocopherol and N6-monomethyl-L-arginine were unable to protect cells from erbstatin-induced death. It is also of interest that the intracellular concentration of H2O2 in SCLC cells was gradually increased by the treatment with erbstatin and that this increase was blocked by treatment with the same dose range of H2O2 scavengers effective in inhibiting erbstatin-induced cell death. These results suggest that H2O2 is the likely intermediate that mediates apoptosis in erbstatin-treated SCLC cells. Indeed, in SCLC cells, H2O2 (and menadione, which increases the intracellular H2O2 concentration) induced DNA fragmentation and cell death. As expected, cell death induced by H2O2 (or menadione) was inhibited by H2O2 scavengers.

An increased level of H2O2 was observed when SCLC cells were treated not only with erbstatin but also with herbimycin A. Therefore, inhibition of tyrosine kinase would cause aberrant generation of H2O2.

Although various enzyme activities are altered by tyrosine phosphorylation, neither catalase nor SOD, which regulate intracellular H2O2
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Fig. 4. Induction by erbstatin of hydrogen peroxide production in Ms-1 cells without changing the SOD and catalase activities or Bcl-2 expression. A. Ms-1 cells were treated with 30 μg/ml erbstatin for the indicated times without or with NAC (2 mM) or GSH (2 mM) in the presence of 5 μM DCFH-DA. B–D, Ms-1 cells were treated with 30 μg/ml erbstatin for 0, 1, 2, or 4 h. SOD (B) and catalase (C) activities were detected on polyacrylamide gels as described in “Materials and Methods.” For the assay of Bcl-2 expression (D), 50 μg of protein of cell lysates were electrophoresed and immunoblotted with anti-Bcl-2 antibody.

concentration, was influenced following tyrosine kinase inhibitor treatment of SCLC cells. Inhibition of tyrosine kinase failed to decrease the Bcl-2 protein level in Ms-1 cells. This result is inconsistent with the recent finding that, in cytokine-dependent lymphocytes, expression of Bcl-2 decreased in the apoptosis induced by treatment with herbimycin A (10). One possible explanation of this discrepancy is that lymphocytes and SCLC cells have different signaling pathways for Bcl-2 expression. It is also still possible that other proteins like Bcl-2 may be involved in induction of apoptosis in SCLC cells. Furthermore, we cannot exclude the possibility that Bcl-2 loses its antiapoptotic potential following the inhibition of tyrosine phosphorylation of Bcl-2.

Tyrosine kinase inhibitor-induced apoptosis requires new protein synthesis, because CHx prevented the DNA fragmentation induced by

Fig. 5. Suppression of erbstatin-induced DNA fragmentation by CHx. Ms-1 cells were labeled with [3H]thymidine (0.5 μCi/ml) for 2 h. Then, the cells were treated with erbstatin (30 μg/ml; A) or H2O2 (0.3 mM; B) for 16 h in the presence or absence of CHx (10 μg/ml). The DNA fragmentation ratio was calculated as described in “Materials and Methods.” Each column indicates the mean of quadruplicate experiments; bars, SD. *, P < 0.01 against the value with erbstatin alone.
eribstatin. We also showed that an increase in $H_2O_2$ following treatment with eribstatin or herbimycin A was also partially eliminated by CHx, indicating that newly synthesized proteins derived from the inhibition of tyrosine kinase, at least in part, impair the regulatory system of $H_2O_2$ generation. Moreover, once $H_2O_2$ is generated, no more new protein synthesis is required for apoptosis, because $H_2O_2$-induced apoptosis was not inhibited by CHx. In many types of apoptosis, CHx is considered to prevent the synthesis of lethal proteins, although not necessarily endonucleases. However, it is still unclear whether the mechanism of eribstatin-induced apoptosis shares the common lethal proteins. Although our findings indicate that tyrosine kinase inhibitor-induced apoptosis in SCLC cells is mediated by $H_2O_2$ via newly synthesized proteins, the signaling pathway leading to the $H_2O_2$ production remains to be studied.

**REFERENCES**


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