Cholera Toxin Triggers Apoptosis in Human Lung Cancer Cell Lines

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Abstract

Cholera toxin (ChT) inhibits signals generated by multiple growth factors in human lung cancer cells, resulting in cell growth inhibition. We now report that ChT triggers apoptosis as shown by DNA fragmentation and activation of caspases cleaving poly(ADP-ribose) polymerase and lamin B. Apoptosis induced by ChT in a small cell lung cancer cell line is not affected by manipulations of intracellular cAMP through preincubation with isobutylmethylxanthine but can be modestly increased through inhibition of protein kinase C with chelerythrine. Thus, apoptosis is actively suppressed in lung cancer cells by a ChT-sensitive-growth regulatory pathway, and these observations may have significant implications in the development of novel strategies for lung cancer treatment.

Introduction

Lung cancer cells commonly coexpress growth factors and growth factor receptors, thus deriving growth support from autocrine/paracrine stimulatory loops (1, 2). ChT inhibits the intracellular calcium pathway, and these observations may have significant implications in the development of novel strategies for lung cancer treatment.

Materials and Methods

Chemicals. Radioactive [2-14C]thymidine (59 mCi/mmol) was obtained from ICN BioMedicals (Costa Mesa, CA). ChT, IBMX, and chelerythrine were purchased from CalBiochem (San Diego, CA). All other chemicals were of reagent grade and purchased either from Sigma Chemical Co. (St. Louis, MO), ICN BioMedicals (Costa Mesa, CA), Boehringer Mannheim (Indianapolis, IN), or Bio-Rad Laboratories (Hercules, CA).

Cell Culture and DNA Labeling. Human SCLC (NCI-H345, NCI-H69, and NCI-H417) and NSCLC (NCI-H226 and NCI-H661) cell lines were obtained from Dr. Adi Gazdar, National Cancer Institute, NIH (Bethesda, MD). Cells were maintained at 37°C in the presence of 5% CO2 in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cell culture products were purchased from Life Technologies, Inc. (Grand Island, NY). For DNA labeling, cells were grown with [14C]thymidine (0.02 μCi/ml) for 24 h and then chased in isotope-free medium overnight prior to drug treatment.

Quantification of DNA Fragmentation by DNA Filter Elution Assay. DNA filter elution assays were designed to monitor and quantitate DNA fragmentation associated with apoptosis (7). Each sample (approximately 0.5 × 106 [14C]thymidine-labeled cells) was loaded onto a protein-adsorbing filter (vinyl/acyrlyc copolymer filters with Metriel membrane, 0.8-μm pore size, and 25-mm diameter; Gelman Sciences, Inc., Ann Arbor, MI) mounted in a Millipore filter holder connected to a 50-ml syringe. Cells were then washed with 5 ml of PBS (fraction 1). As soon as the washing solution had dripped through by gravity, lysis was performed with 5 ml of lysis solution [2% SDS, 100 mM glycine, and 25 mM EDTA (pH 10.0; fraction 2)]. After the lysis had dripped through by gravity, it was washed from the filters with 5 ml of 0.02 M EDTA (pH 10.0; fraction 3). The filter was then processed as described (fraction 4; Ref. 7). Briefly, it was placed in a scintillation vial to which 0.4 ml of 1 N HCl was added. Following incubation for 1 h at 60°C to depurate the DNA, 2.5 ml of 0.4 mM NaOH were added and allowed to stand for 1 h at room temperature to release the labeled DNA from the filters. Radioactivity was counted by liquid scintillation spectrometry in each fraction. DNA fragmentation was determined as the fraction of DNA in the loading wash fraction + lysis fraction + EDTA wash fraction relative to total DNA (loading wash + lysis + EDTA wash + filter). Results are expressed as the percentage of DNA fragmented in treated cells compared to DNA fragmented in control untreated cells (background) using the formula:

\[
\frac{F - F_0}{1 - F_0} \times 100
\]

where F and F0 represent DNA fragmentation in treated and control cells, respectively.

Analysis of DNA Fragmentation by Agarose Gel Electrophoresis. At specified times during ChT treatment, cellular DNA was extracted by a salting-out procedure as described previously (7). Electrophoresis was performed in 1.6% agarose gel in Tris-borate buffer (pH 8.0) at 2.5 V/cm for 15 h. Following electrophoresis, DNA was visualized by ethidium bromide staining.

Protein Extraction and Immunobots. Protein extraction and Western blot were performed according to previously described protocols, with small modifications (8). Briefly, at specified times after treatment, cells were washed twice with ice-cold PBS and then lysed for 1 h at 4°C in buffer containing 50 mM Heps (pH 7.5), 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 5 mM sodium fluoride, 10 mM Na PPh, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylylsulfonyl fluoride, and a mixture of protease inhibitors (Complete; Boehringer Mannheim). Lysates were centrifuged at 14,000 rpm for 15 min at 4°C, and supernatants were collected. Protein concentrations were determined using the Bradford assay with BSA as standard (Bio-Rad, Hercules CA). SDS-PAGE was performed using 1.0-mm-thick slab gels (12%) and proteins transferred to Immobilon-P membrane (Millipore, Bedford MA). Affinity-purified antibodies to human Bax and Bcl-2 proteins were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and antibodies to human PARP and lamin B proteins were obtained from Boehringer Mannheim and Calbiochem, respectively. Enhanced chemiluminescence detection of peroxidase-labeled secondary antibodies was performed using Amersham Life Science reagents.

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4 The abbreviations used are: ChT, cholera toxin; IBMX, isobutylmethylxanthine; SCLC, small cell lung carcinoma; NSCLC, non-small cell lung carcinoma; PARP, poly(ADP-ribose) polymerase; PKC, protein kinase C.
Results and Discussion

DNA Fragmentation Induced by ChT in a Series of SCLC and NSCLC Cell Lines. The differential effect of ChT on the growth of a series of SCLC and NSCLC cell lines was reported previously (4). For SCLC cell lines, a correlation has been established between resistance to ChT and absent expression of G_{M1} ganglioside, the cell surface binding site for ChT. However, for NSCLC, ChT resistance is observed in G_{M1} ganglioside-positive cells (4). In this study, we have examined further the relationship between growth inhibition induced by ChT and induction of DNA fragmentation associated with apoptosis in some ChT-sensitive and -resistant cell lines. We routinely used a filter DNA elution assay to monitor and quantitate kinetics of DNA fragmentation in cells undergoing apoptosis (7). Treatments with ChT at various concentrations induced DNA fragmentation in the ChT-sensitive SCLC NCI-H345, NCI-H69, and NSCLC NCI-H226 cell lines, whereas no DNA fragmentation was detected in the ChT-resistant SCLC NCI-H417 and NSCLC NCI-H661 cell lines (Fig. 1).

Maximal DNA fragmentation was achieved at 2.5 \( \mu \)g/ml ChT, and the extent of DNA fragmentation increased with time. However, in human lung carcinoma cell lines resistant to ChT, such as the SCLC NCI-H417 and NSCLC NCI-H661 cells, no significant DNA fragmentation was observed (Fig. 1). These results indicated a direct relation between the reported differential effect of ChT on cell growth and the extent of DNA fragmentation in a series of human lung carcinoma lines (4) and the induction of DNA fragmentation in sensitive cells.

Apoptosis is an active mode of cell death that is defined by characteristic cellular morphological changes associated with digestion of chromatin that can be visualized as an oligonucleosome-sized DNA ladder on an ethidium bromide-stained agarose gel (9, 10). To confirm the specificity of the DNA fragmentation observed by the filter assay, we visualized the intensity of the oligonucleosome-sized DNA fragments caused by ChT in the SCLC NCI-H345 lines by agarose gel electrophoresis analysis at specified times during drug treatment (Fig. 2A). Activation of apoptotic proteases is known to be a critical step in dying cells. Series of caspases were reported to play a central role in executing apoptosis (11, 12). The caspases are separated into three subfamilies based on their degrees of homology and show specificity in their substrates (13). Kinetics of PARP and lamin B cleavage, known substrates of caspase 3-like activity (14, 15), were monitored in control and ChT-treated SCLC NCI-H345 lines by Western blot analysis. Fig. 2B shows that native lamin B protein (\( M_r \) 67,000) is cleaved into a typical fragment of \( M_r \) 46,000 in ChT-treated cells. Similarly, native PARP protein (\( M_r \) 113,000) is cleaved into a typical fragment of \( M_r \) 89,000. These results indicate that ChT induces apoptosis in the sensitive SCLC NCI-H345 cells.

Modulation of ChT-induced DNA Fragmentation in SCLC NCI-H345 Cells. One of the first early responses of target cells to ChT is an elevation of intracellular cAMP (3). However, cAMP does not contribute to the effects of ChT on growth factor-stimulated activation of phospholipase C and calcium signaling, nor to the growth inhibition induced by ChT. To investigate whether cAMP could contribute to the apoptosis induced by ChT, cells were incubated 5 min with IBMX, an inhibitor of phosphodiesterase, to potentiate levels of cAMP achieved by ChT treatment (4). As shown in Fig. 3, pretreatment of cells at 0.5 mM IBMX did not significantly modulate the extent of DNA fragmentation in ChT-treated SCLC NCI-
H345 cells. These results suggest that the cAMP-signaling pathway is not implicated in ChT-induced apoptosis, consistent with the prior evidence for the independence of ChT effects from cAMP in human lung cancer cell lines. Similarly, we investigated the effect of chelerythrine, an inhibitor of the PKC catalytic domain, upon ChT-induced apoptosis. Pretreatment of NCI-H345 cells with 2.0 μM chelerythrine prior to ChT treatment slightly increased the amount of DNA fragmentation compared to NCI-H345 cells treated with ChT alone (Fig. 3). These results suggest that modulation of PKC in combination with ChT may accelerate or increase the level of DNA fragmentation in these cells. This is in agreement with prior observations demonstrating that ChT can inhibit bombesin-stimulated activation of phospholipase C in NCI-H345 cells (16). Given that inhibition of PKC amplifies apoptosis triggered by ChT in these cells, it is likely that the ChT-sensitive pathway we have described also uses PKC as an effector.

Recent studies have suggested that ordered balance within the ratio of effector:repressor proteins controlling apoptosis may be important checkpoints regulating apoptosis (17). Among the various members of the Bcl gene family, the ratio of Bax:Bcl-2 proteins in human tumors has been related to cell susceptibility to undergoing apoptosis (18–20). Thus, to evaluate change in the expression level of these proteins, kinetics of human Bcl-2 and Bax proteins were determined in control and ChT-treated NCI-H345 cells. Western blot analysis indicated that the level of expression of either Bcl-2 or Bax protein remained unchanged for at least 48 h after ChT treatment (Fig. 4). At 72 h after drug treatment, a slight increase in Bax expression could be detected (Fig. 4). These results indicate that variation in the expression level of these proteins did not precede the activation of DNA fragmentation but may accompany occurrence of apoptosis in ChT-treated NCI-H345 cells.

Recently, it has been reported that inhibition of neuropeptide-stimulated tyrosine phosphorylation and tyrosine kinase activity could stimulate apoptosis in lung cancer cells (21). We show here that ChT, which inhibits signals generated by multiple growth factors in human lung cancer cells, triggers apoptosis in these cells. Taken together, our observations suggest that apoptosis can be suppressed actively in lung cancer cells by a ChT-sensitive growth-regulatory pathway. This pathway is probably normally activated by neuropeptides, such as bombesin, acting through autocrine/paracrine growth-regulatory loops. Our observations are consistent with those reported previously in other tumor cell systems in which insulin-like growth factor 1 stimulation acting through a receptor tyrosine kinase also suppressed what would otherwise be a spontaneous tendency for cells to undergo apoptosis (6). It thus appears that the maintenance of trophic stimulation by growth factors acting through a variety of receptor classes and effector pathways is a central feature of cancer cell growth. Furthermore, expression of these receptors and associated growth factors may be also important for decreasing tumor sensitivity to chemotherapy-induced apoptosis (22). Therefore, innovative anti-growth factor cancer therapies may be rewarding in tumor cell types in which such pathways can be demonstrated to actively suppress apoptosis.

References

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