Cotylenin A, a Differentiation-inducing Agent, and IFN-α Cooperatively Induce Apoptosis and Have an Antitumor Effect on Human Non-Small Cell Lung Carcinoma Cells in Nude Mice

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ABSTRACT

Cotylenin A, a novel inducer of the differentiation of leukemia cells, and IFN-α synergistically inhibited the growth of and induced apoptosis in several human non-small cell lung carcinoma cell lines. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and its receptor DR5 were the early genes induced by the combination of cotylenin A and IFNα in lung carcinoma cells. Neutralizing antibody to TRAIL inhibited apoptosis, suggesting that cotylenin A and IFNα cooperatively induced apoptosis through the TRAIL signaling system. This combined treatment preferentially induced apoptosis in human lung cancer cells while sparing normal lung epithelial cells and significantly inhibited the growth of human lung cancer cells as xenografts without apparent adverse effects, suggesting that this combination may have therapeutic value in treating lung cancer.

INTRODUCTION

NSCLC is one of the most common malignant diseases in the world. Surgical resection is the only treatment modality with a reasonable chance of offering cure when applied to appropriately selected patients. Only ~40% of patients have resectable disease, and only one-quarter of these (10–12% overall) are still alive at 5 years and apparently cured of their disease. Chemotherapy is reserved solely for patients with advanced stages of NSCLC and until now has only brought marginal benefits (1). Efforts are underway to optimize chemotherapy strategies and discover new agents. Attempts to translate recent findings regarding the biology of lung cancer into therapeutic strategies, such as the use of biological response modifiers, monoclonal antibodies, and inhibitors of signal transduction of oncoproteins, may some day lead to significant progress.

IFNs are pleiotropic cytokines that block viral infection, inhibit cell proliferation, induce apoptosis, and modulate cell differentiation (2). IFNα has therapeutic activity as a single agent in some types of hematological malignancies but is less effective in the therapy of solid tumors, including NSCLC (2–4). To overcome this resistance, various therapeutic approaches have been developed. The combination of IFNα with conventional chemotherapeutic agents has been reported to be effective at inducing tumor regression in some tumors, including NSCLC (5–7). However, the precise mechanisms of action and optimal dosing and sequencing in combination with chemotherapy are unclear. Additional studies are needed to more clearly define the role of IFNα as a modulator of cytotoxic chemotherapy agents.

Differential-inducing agents can alter the phenotype of cancer cells, including their drug sensitivity (8, 9). Retinoids in combination with IFNs are highly effective against several malignancies (10–12) but do not affect the sensitivity of NSCLC cells to IFNα (13). Furthermore, although some differentiation-inducing agents effectively enhance the sensitivity of lung cancer cells to IFNα with regard to the inhibition of cell proliferation, retinoids do not (14). Although IFNα alone only slightly inhibited the growth of lung cancer cells at high concentrations, combined treatment with IFNα and suboptimal concentrations of some differentiation-inducing agents greatly reduced the growth of a variety of human lung cancer cell lines both in vitro and in vivo (14). Although this is a promising approach to lung cancer therapy, DMSO and sodium butyrate are not suitable for use in the treatment of patients with NSCLC. In the present investigation, we examined the synergistic effects of various differentiation-inducing agents and IFNα on the growth of lung cancer cells to identify the most potent and clinically applicable drugs. The most effective agent was cotylenin A, a novel inducer of differentiation of myeloid leukemia (15, 16). Cotylenin A, which has a novel fusicoccane-diterpene glycoside with the complex sugar moiety, was isolated as a plant growth regulator and has been shown to affect several physiological processes in higher plants (17, 18). Cotylenin A also affected the differentiation of leukemic cells that were freshly isolated from acute myelogenous leukemia patients in primary culture (19). It significantly stimulated both the functional and morphological differentiation of leukemia cells in 9 of 12 cases. This differentiation-inducing activity was more potent than those of all-trans retinoic acid and 1α,25-dihydroxyvitamin D3 (19). Because cotylenin A is potent at stimulating differentiation in vitro, it may have therapeutic effects in experimental models of leukemia and acute myelogenous leukemia patients. Injection of the human promyelocytic leukemia cell line NB4 into mice with severe combined immunodeficiency resulted in the death of all mice caused by leukemia. Administration of cotylenin A significantly prolonged the survival of mice inoculated with retinoid-sensitive and -resistant NB4 cells, and no appreciable adverse effects were observed in the experiment (20). These results suggest that cotylenin A may be useful in therapy for leukemia and some other malignancies. Therefore, in the present study, we sought to clarify the synergistic effect of cotylenin A and IFNα on human lung carcinoma cells and to examine the therapeutic effects on xenografts of human lung carcinoma cells.

MATERIALS AND METHODS

Chemicals. Cotylenin A was purified from the culture filtrate of Cladosporium fungus sp. 501–7W by flash chromatography on silica gel with >99% purity (17, 18). A stock solution of cotylenin A was prepared in absolute ethanol at 20 mg/ml. Human natural IFNα (Sumiferon) was a kind gift from Sumitomo Pharmaceuticals (Tokyo, Japan). MTT, Fas ligand, and anticancer drugs were obtained from Sigma Chemical (St. Louis, MO). DMSO, recombinant human TRAIL, recombinant human TNFα, and Na-butyrate were obtained from Wako Pure Chemicals (Osaka, Japan). TGFβ, caspase inhibitors, and antihuman TRAIL antibody were obtained from R&D Systems (Minneapolis, MN).
Cells and Cell Culture. Human lung carcinoma cell lines were maintained in RPMI 1640 supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO₂ in air (14). A549 was established from a human lung carcinoma with properties of type II alveolar epithelial cells. PC9 and PC14 were well- and poorly differentiated adenocarcinoma cell lines, respectively.

Human normal bronchial epithelial and SAE cells, which were derived from human lung bronchus and the small airway of healthy donors, respectively, were purchased from Clonetics (San Diego, CA) and grown in serum-free Bronchial Epithelial Cell and Small Airway Epithelial Cell Growth Medium Bullet kits (Clonetics), respectively.

Assay of Cell Growth and Apoptosis. The cells were seeded at 1 x 10⁴/ml in a 24-well multidi. After culture with or without the test compounds for the indicated times, viable cells were examined by the modified MTT assay. Briefly, 100 µl of MTT solution (1 mg/ml in PBS) were added to each well. After incubation with MTT for 4 h, the cells were centrifuged at 1000 x g for 10 min. The precipitates were dissolved in 1 ml of DMSO, and their absorption at 560 nm was determined. Assay of the cumulative cell number was determined as described elsewhere (21). The cellular DNA content was analyzed using propidium iodide-stained nuclei (21). Caspase activity in intact cells was measured using PhiPhiLux by flow cytometry analysis according to the manufacturer's instructions (OncoImmunity, Inc., Gaithersburg, MD).

Analysis of TRAIL-, DR4-, and DR5-positive Cells. We detected the expression of TRAIL and its receptors by flow cytometry. Cells were suspended in 100 µl of cold PBS with 2.5% FBS and incubated with antihuman TRAIL, DR4, or DR5 IgG (Cayman Chemical, Ann Arbor, MI) for 30 min on ice. Cells were then washed with PBS with 2.5% FBS and incubated with 100 µl of fluorescein-isothiocyanate-conjugated goat antirabbit IgG antibody (Beckman-Coulter Electronics, Miami, FL). The percentage of fluorescence-positive cells was determined by setting gates to exclude ≥99% positive cells (fluorescent) in the isotype control.

Gene Expression Analysis by RT-PCR. Total RNA was extracted using Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Total RNA (1 µg) from lung cancer cells was converted to first-strand cDNA primed with random hexamer in a 20-µl reaction using an RNA PCR kit (Takara Shuzo Co., Ltd., Tokyo, Japan), and 4 µl of this reaction were used as a template in the PCR. The oligonucleotides used in PCR amplification were as described elsewhere (22, 23), and a quantitative RT-PCR reaction was performed as described in the literature (24).

Transplantation of Lung Cancer Cells into Nude Mice and Treatment. Female athymic nude mice with a BALB/c genetic background were supplied by CLEA Japan (Tokyo, Japan). They were housed under specified pathogen-free conditions. The in vivo experiments were performed in accordance with the guidelines of our institute (Guide for Animal Experimentation, Saitama Cancer Center). Mice were inoculated s.c. with 6 x 10⁴ A549 cells in 0.2 ml of PBS in 100 µl of Matrigel (BD Biosciences, San Diego, CA).

RESULTS

Combined Effects of IFNα and Various Drugs on the Growth of NSCLC Cells. To measure the effects of various drugs on the growth of lung carcinoma A549 cells, the number of viable cells was determined by the MTT assay after 6 days of exposure to various concentrations of drugs with or without 300 IU/ml IFNα. The growth-inhibiting effects of the drugs were examined by determining the concentrations of drugs required to reduce the cell number to one-half of that in untreated cells (IC₅₀). The sensitivity to anticancer agents, such as 5-fluorouracil, cis-platin, or doxorubicin, was not affected by IFNα, whereas the sensitivity to hydroxyurea was significantly enhanced (Table 1). Most of the differentiation-inducing agents for myeloid leukemia cells were not toxic toward the lung carcinoma cell line when used within a range of concentrations that were effective at inducing the differentiation of human myeloid leukemia cells, suggesting that the lung cancer cells were less sensitive to agents that inhibited cell growth. Retinoids and vitamin D₃ did not affect the growth of A549 cells even at high concentrations (Table 1). The growth-inhibitory effect of DMSO was significantly enhanced by IFNα, although IFNα alone hardly inhibited cell growth (Table 1). We next examined the effects of >50 compounds that induce the differentiation of myeloid leukemia cells (25) and found that although the effects of some agents were strongly affected by IFNα, the effects of other agents were not. Among the differentiation-inducing agents tested, the sensitivity of lung cells to cotylenin A was most strongly affected by IFNα (Table 1). The synergistic effects of cotylenin A and IFNα were also observed in other lung carcinoma cell lines, although the sensitivity of lung carcinoma cell lines to IFNα varied among the cell lines (Fig. 1 and Table 2).

Next, we examined the combined effects of cotylenin A and various cytokines on the growth of A549 cells. Unlike IFNα, IFNγ did not affect cotylenin A-induced growth inhibition (Fig. 2b). TNFα and TGFβ also showed cooperative effects with cotylenin A in the inhibition of cell proliferation (Fig. 2c, d), whereas other cytokines, such as interleukin 1, interleukin 4, leukemia-inhibitory factor, granulocyte macrophage colony-stimulating factor, granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor, and keratinocyte growth factor, did not show such cooperation with cotylenin A (data not shown). IFNα was the most potent cytokine at inhibiting cell growth in the presence of cotylenin A. These results suggest that the combination of cotylenin A and IFNα is the most potent for inhibiting the growth of human lung cancer cells.

**Table 1**: Potentiation of the growth-inhibitory activities of various agents in human lung carcinoma A549 cells by IFNα

<table>
<thead>
<tr>
<th>Compound</th>
<th>Growth inhibition (IC₅₀)</th>
<th>Ratio (−/+)</th>
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<tbody>
<tr>
<td>IFNα</td>
<td>+</td>
<td>IFNα</td>
</tr>
<tr>
<td>5-Fluorouracil (µg/ml)</td>
<td>0.81 ± 0.06</td>
<td>0.59 ± 0.04</td>
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<tr>
<td>Hydroxyurea (µM)</td>
<td>407 ± 38</td>
<td>149 ± 11</td>
</tr>
<tr>
<td>Doxorubicin (ng/ml)</td>
<td>31.4 ± 2.8</td>
<td>22.8 ± 1.9</td>
</tr>
<tr>
<td>Cis-platin (µg/ml)</td>
<td>312 ± 3.0</td>
<td>259 ± 22</td>
</tr>
<tr>
<td>Etoposide (µg/ml)</td>
<td>5.13 ± 0.3</td>
<td>3.03 ± 0.2</td>
</tr>
<tr>
<td>Camptothecin (µg/ml)</td>
<td>124 ± 8.1</td>
<td>72.2 ± 4.8</td>
</tr>
<tr>
<td>Actinomycin D (ng/ml)</td>
<td>0.31 ± 0.02</td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td>All-trans retinoic acid (µM)</td>
<td>&gt;36</td>
<td>&gt;36</td>
</tr>
<tr>
<td>13-Cis retinoic acid (µM)</td>
<td>&gt;36</td>
<td>&gt;36</td>
</tr>
<tr>
<td>1,25-Dihydroxyvitamin D₃ (ng/ml)</td>
<td>&gt;90</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Cotylenin A (µg/ml)</td>
<td>15.4 ± 1.1</td>
<td>1.18 ± 0.01</td>
</tr>
<tr>
<td>Sodium butyrate (µM)</td>
<td>521 ± 43</td>
<td>164 ± 11</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (mM)</td>
<td>257 ± 21</td>
<td>78.2 ± 7.3</td>
</tr>
<tr>
<td>Hexamethylene bisacetamide (mM)</td>
<td>12.7 ± 1.1</td>
<td>4.4 ± 0.4</td>
</tr>
<tr>
<td>Isopentenyladenine (µg/ml)</td>
<td>86.1 ± 8.2</td>
<td>69.3 ± 5.9</td>
</tr>
<tr>
<td>8-Cl-CdAMP (µM)</td>
<td>24.7 ± 1.9</td>
<td>18.8 ± 1.6</td>
</tr>
<tr>
<td>Bufalin (nM)</td>
<td>1.6 ± 0.1</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>Trichostatin A (ng/ml)</td>
<td>71.5 ± 7.6</td>
<td>48.2 ± 4.1</td>
</tr>
<tr>
<td>Genistein (µg/ml)</td>
<td>8.2 ± 0.7</td>
<td>6.3 ± 0.6</td>
</tr>
<tr>
<td>Wortmannin (µM)</td>
<td>4.9 ± 0.5</td>
<td>3.2 ± 0.3</td>
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a Cells were incubated with various concentrations of the test compounds in the presence or absence of 600 IU/ml IFNα for 6 days. Ratio (−/+), IC₅₀ without IFNα/IC₅₀ with IFNα. The values are the mean ± SD of four determinations.
cell growth in a concentration-dependent manner. Cotylenin A alone induced G1 arrest, but not apoptosis, even at a high concentration (Fig. 3A). Combined treatment with 300 IU/ml IFNα plus 4 μg/ml cotylenin A caused significant growth inhibition, and this effect was similar to that with 24 μg/ml cotylenin A alone (Fig. 4). When the cells were treated with cotylenin A and IFNα for 7 days, and then washed and cultured without the drugs, cell growth was greatly inhibited at day 16, indicating that the growth-inhibitory effect of cotylenin A plus IFNα was irreversible in long-term culture. On the other hand, cotylenin A-treated cells began to grow within a few days after removal of the drug (Fig. 4). These results were consistent with the finding that the combination of cotylenin A and IFNα induced apoptosis, whereas a high concentration of cotylenin A induced G1 arrest in A549 cells (Fig. 3), and suggest that this combined treatment may have therapeutic value in the chemotherapies of some lung cancers.

Fas and/or TRAIL signaling pathways are involved in IFN-induced apoptosis in some malignant cells (26–29). Therefore, we examined the effects of Fas ligand and TRAIL on the growth of A549 cells in the presence of IFNα or cotylenin A. The combination of IFNα and Fas ligand or TRAIL scarcely inhibited the proliferation of A549 cells (data not shown), whereas cotylenin A cooperated with TRAIL at inhibiting cell growth (Fig. 5a). However, Fas ligand did not affect the growth inhibition induced by cotylenin A (Fig. 5b). Similar results were obtained when the cells were treated with anti-Fas monoclonal antibody CH-11, which was highly effective at inducing apoptosis in lymphoid cells. Although TRAIL significantly enhanced the growth inhibition induced by DMSO, the enhancing effects were less than those of cotylenin A (Fig. 5c). Similar results were observed when
TRAIL selectively induces apo-
of Normal Lung Epithelial Cells. T
form of caspase-8 inhibitor on the growth inhi
ation of caspase-8 (30, 31). Therefore, we examined t
expression of DR5 significantly increased after treatment with low levels of TRAIL and its receptors DR4 and DR5 (Fig. 6). The cell 
examined by flow cytometric analysis. Untreated cells expressed very

Fig. 4. Proliferation of A549 cells in long-term culture with IFNα and cotylenin A. Cells were cultured without (○) or with 300 IU/ml IFNα (□), 4 μg/ml cotylenin A (△), 24 μg/ml cotylenin A (●), or 300 IU/ml IFNα plus 4 μg/ml cotylenin A (■). □ and ○ represent cells that were cultured with 300 IU/ml IFNα plus 4 μg/ml cotylenin A and 24 μg/ml cotylenin A for 7 days and then washed and cultured further without drugs, respectively. The culture medium was replaced by fresh medium at least once every 3 days. The cell density was kept at 1–8 × 10^5/ml. The values are means of four separate experiments.

cells were treated with hydroxyurea or doxorubicin (Fig. 5c). Treat-
ment with anti-TRAIL antibody partially blocked the apoptosis med-
iated by cotylenin A plus IFNα (Fig. 5d). These results suggest that a TRAIL signaling pathway plays a role in the apoptosis induced by cotylenin A plus IFNα.

The expression of TRAIL and its receptors in A549 cells was exa
mained by flow cytometric analysis. Untreated cells expressed very low levels of TRAIL and its receptors DR4 and DR5 (Fig. 6). The cell 
surface expression of DR5 significantly increased after treatment with cotylenin A plus IFNα but not after treatment with cotylenin A or IFNα alone. Although the expression of TRAIL was dose dependently increased by high concentrations of IFNα, no significant increase in TRAIL expression was observed on treatment with 300 IU/ml IFNα. However, this concentration of IFNα significantly enhanced the cotylenin A-induced expression of TRAIL (Fig. 6). The changes in DR4 expression were minimal in lung carcinoma cells treated with cotylenin A and/or IFNα.

TRAIL-induced apoptosis is known to be mediated by the activa-
tion of caspase-8 (30, 31). Therefore, we examined the effect of a caspase-8 inhibitor on the growth inhibition induced by cotylenin A plus IFNα (Fig. 5e). Caspase-8 inhibitor significantly blocked growth inhibition, whereas caspase-4 inhibitor had only modest effects, and other caspase inhibitors, such as inhibitors of caspase-1 and -9, did not essentially affect growth inhibition (data not shown), suggesting that the activation of caspase-8 plays a role in the induction of apoptosis.

The Combined Effects of IFNα and Cotylenin A on the Growth of Normal Lung Epithelial Cells. TRAIL selectively induces apo-
ptosis in some cancer cells while sparing normal human epithelial cells (32–34). Therefore, we compared the combined effects of IFNα and cotylenin A in two types of normal human lung epithelial cells, normal human bronchial epithelial and SAE cells, which were derived from human lung bronchus and the small airway of healthy donors, respectively, with those in lung cancer cell lines (Fig. 7). The optimal culture conditions for the normal cells were serum-free medium, whereas lung cancer cells grow in the presence of 10% FBS. In serum-free culture conditions, cells were more sensitive to the com-
be a dose-escalating effect was not observed in the thera-
peutic experiments (20). Therefore, we administered 100 μg/mouse cotylenin A, which had no appreciable adverse effects on mice, even in the presence of IFNα. The mice were injected with 3 × 10^6 IU of IFNα every day. This dose is equivalent to ~3 × 10^5 IU/m² daily in humans using the calculations of Freireich et al. (35). The combined treatment significantly inhibited the growth of PC14 cells as xenogra-
figs (Fig. 9). At day 12 after treatment, the mean tumor volumes of untreated, cotylenin A-, IFNα-, and cotylenin A plus IFNα-treated nude mice were 520.4 ± 82.7, 318.5 ± 65.4, 391.6 ± 61.5, and
21.8 ± 20.6, respectively. Although cotylenin A and IFNα each significantly retarded tumor growth (P < 0.05), combined treatment induced tumor regression. The treatment was continued for 12 days and then stopped, with a follow-up on day 26. All of the untreated mice had a large tumor burden at day 26. On the other hand, 50% of the treated mice escaped from the disease (13 of 20 mice), and the rest had only a small tumor burden, suggesting that the therapeutic effects were still maintained after treatment was terminated. These results indicate that the combination of cotylenin A and IFNα is more effective therapeutically than treatment with cotylenin A or IFNα alone, and the combined treatment had a significant antitumor effect (P < 0.001).

Fig. 5. Combined effects of cotylenin A and TRAIL (a) or cotylenin A and Fas ligand (b) on the growth of A549 cells. Cells were cultured with TRAIL or Fas ligand in the presence of 0 (●), 4 (▲), 8 (▲), 12 (▲), or 16 (~) µg/ml cotylenin A for 6 days. Combined effects of drugs and TRAIL on the growth of A549 cells (c). Cells were cultured with various concentrations of TRAIL in the presence of 0 (●), 78 (●), 140 (▲), 210 (▲), or 280 (~) µM cotylenin A (left); 0 (●), 0.1 (●), 0.3 (▲), or 0.9 (~) µM hydroxyurea (middle); and 0 (●), 5 (●), 10 (▲), or 20 (~) ng/ml daunorubicin (DXR) for 6 days (right). Effect of anti-TRAIL antibody (d) or caspase-8 inhibitor (e) on the growth inhibition induced by cotylenin A plus IFNα. Cells were simultaneously treated without (open bar) or with 0.3 (striped bar) or 1 (dotted bar) µg/ml anti-TRAIL antibody or 20 (striped bar) or 60 (dotted bar) µM caspase-8 inhibitor in the presence of 300 IU/ml IFNα plus cotylenin A for 6 days. Viable cell number was determined by the MTT assay. The values are mean ± SD of four determinations.

Fig. 6. Combined treatment with cotylenin A and IFNα up-regulates TRAIL and DR5 expression in A549 cells. Cells were treated with various concentrations of cotylenin A in the presence of 0 (●), 150 (●), 300 (▲), or 450 (~) IU/ml IFNα for 4 days. Expression was assayed by flow cytometry. The values are mean ± SD of four determinations.
DISCUSSION

IFNα alone had no significant effects on the growth or viability of human lung cancer cell lines, even at 2,000 IU/ml. However, we found previously that several human lung cancer cell lines similarly expressed IFNα receptors (3,000–10,000 receptors/cell) with high affinity, and activation of the transcription factor ISGF-3, which has been shown to be required for the transcriptional activation of IFN-induced genes, was induced by IFNα alone in lung carcinoma PC9 cells (14). Moreover, IFNα rapidly induced the expression of some IFNα-inducible genes, such as IRF-1 and PML, in PC9 cells (data not shown). The present study also showed that the expression of TRAIL mRNA was induced by IFNα alone (Fig. 8), suggesting that resistance and its restoration by differentiation-inducing agents, such as cotylenin A and DMSO, in some lung carcinoma cells act downstream of the activation of ISGF-3. Although IFNα alone up-regulated the expression of some genes associated with apoptosis and growth inhibition, it might be inadequate for activating some important genes to exert significant effects on apoptosis. Expression of the death receptor DR5 was not essentially induced by IFNα or cotylenin A alone, but combined treatment greatly up-regulated its mRNA and cell surface expression in lung cancer cells. Wild-type p53 has been shown to up-regulate DR5 gene expression (36). However, expression of DR5 protein expression in A549 cells was not up-regulated by some anticancer drugs that up-regulate p53 protein expression, and the combined effects of cotylenin A and IFNα were observed in both cancer cells with wild-type and mutant/deleted p53. A receptor of fusicoccin, closely related to cotylenin A, has been reported to be a member of the family of 14-3-3 proteins that are commonly found in a huge array of signaling and regulatory pathways (37). A special subfamily of 14-3-3 proteins may bind cotylenin A and affect the interaction with some signaling molecules, including ISGF-3. This modification may lead to up-regulation of DR5 gene expression. However, further investigation is required to explain how up-regulation of DR5 is related to its effects on the 14-3-3 signaling pathway.

To investigate the role of IFNα in the induction of apoptosis in human lung carcinoma cells, we examined the effects of IFNα and/or...
cotylenin A on the expression of caspases, Apaf-1, and bcl-2 family genes by RT-PCR. The expression of caspase-4 mRNA was induced by treatment with IFNα alone but not by treatment with cotylenin A. The combination of IFNα and cotylenin A did not cause an additional increase in the expression of caspase-4 mRNA. However, treatment with an inhibitor of caspase-4 did not affect the apoptosis induced by IFNα and cotylenin A (data not shown). The expression of other caspase, Apaf-1 and bcl-2 family genes was unchanged by IFNα and/or cotylenin A (data not shown). IFNα alone significantly induced the expression of TRAIL mRNA, and the combination of cotylenin A and IFNα caused an additional increase in expression. The gene expression of the TRAIL receptor DR5 was greatly induced by cotylenin A, a plant growth regulator. Br. J. Haematol., 81: 379, 1970.

Although natural retinoids did not affect the growth of NSCLC cells, even in the presence of IFNα, some synthetic retinoids might be effective in synergism with IFNα. CD437 (6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid) induces gene expression of DR4 and DR5 and then the apoptosis of NSCLC cells (38). However, normal lung epithelial cells are less sensitive to the retinoid. CD437 and IFNα might cooperatively induce apoptosis in lung cancer cells. Some triterpenoids induce apoptosis of several tumor cell lines, including NSCLC (39, 40). Because cotylenin A is a diterpenoid and resembling in structure to some extent, the antitumor triterpenoids may also cooperate with IFNα in inducing apoptosis of cancer cells.

Recent published results indicate that TRAIL and chemotherapeutic drugs act synergistically to kill cancer cells (22, 41). In the present study, the cells were more sensitive to the combination of cotylenin A and IFNα than to chemotherapeutic drugs and TRAIL. We did not investigate the maximal tolerable dose or dose-limiting toxicity of cotylenin A in the absence or presence of IFNα, because the maximal concentration of cotylenin A was 100 μg/0.2 ml saline. This treatment has no apparent effects on mice (body weight and behavior). Potent derivatives of cotylenin A that are readily soluble in saline will be required to further develop this therapeutic strategy.
Cotylenin A, a Differentiation-inducing Agent, and IFN-α Cooperatively Induce Apoptosis and Have an Antitumor Effect on Human Non-Small Cell Lung Carcinoma Cells in Nude Mice

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