Abstract. Ursolic acid is a triterpenoid reported to inhibit the invasion of cancer cells. In this study, there was a significant increase in the gene expression of matrix metalloproteinase (MMP)-1, -2, -3, -9 and -10 in H460 cells after treatment with 10 μM ursolic acid for 24 h. Under these experimental conditions, it was found that ursolic acid induced H460 cell apoptosis. These results indicated that matrix metalloproteinase family members are involved not only in invasion, but also in apoptosis of cancer cells. It has been suggested that ursolic acid acts via a glucocorticoid receptor in the regulation of MMP. Our study also demonstrated that the localization of glucocorticoid receptor in the cytosol might be an important factor of MMP up-regulation during ursolic acid-induced H460 cell apoptosis. Ursolic acid induced a typical apoptosis on H460 cells, which was characterized by the activation of caspase-3, nuclear morphological changes and DNA fragmentation.

Ursolic acid is a pentacyclic triterpenoid compound and the major component of some oriental and traditional medicine herbs (e.g., Hedyotis diffusa, Eribotrya japonica and Ligustrum lucidum Ait) wildly distributed all over the world. There is a growing interest in the elucidation of the biological roles of ursolic acid. Since ursolic acid is relatively non-toxic, many investigations have focused especially on its anti-tumor activity (1-4). Ursolic acid has been shown to act at various stages of tumor development, including inhibition of tumorigenesis, inhibition of tumor promotion and induction of tumor cell differentiation. Ursolic acid has an inhibitory effect on tumor cell proliferation through cell-cycle arrest or a mitochondrial intrinsic pathway (1, 2). Harmand et al. demonstrated that ursolic acid induces apoptosis through caspase-3 activation and cell cycle arrest in HaCat cells (3). It was also demonstrated that ursolic acid has an anti-invasive activity in the human fibrosarcoma cell line by reducing the expression of matrix metalloproteinase (MMP)-9 (4).

Matrix metalloproteinases (MMPs), a family of endopeptidases with the ability to degrade ECM proteins, play a fundamental role in inflammation, tissue remodelling, angiogenesis, wound healing, tumor invasion and metastatic progression (5, 6). MMPs can be secreted in a latent form and subsequently processed to active species, but they can also constitute integral membrane proteins, the membrane-type MMPs (MT-MMP) (5-7). Several soluble MMPs involved in the degradation of collagens, laminins and fibronectin are produced by cancer cells, including MMP-8, MMP-9 and MMP-13, raising the possibility that they might contribute to their invasion across basement membranes and interstitial tissues (8-11). MMP members were found to play a key role in cell apoptosis. During apoptosis, MMP members were required for remodelling of the cell matrix and cell-to-cell contact (12).

Apoptosis is a major form of cell death and is associated with characteristic morphological changes including the formation of membrane blebs and apoptotic bodies, chromatin and nuclear condensation, and DNA fragmentation. It is widely accepted that there are two principal pathways of apoptosis, namely caspase-dependent and -independent. The caspases, a family of cysteine proteases, hence play a critical role during apoptosis. There are at least two major mechanisms by which a caspase cascade resulting in the activation of effector caspase-3 may...
be initiated by the most apical caspase, one involves caspase-8 and the other caspase-9 (13, 14). Therefore, the activation of caspase-3 is necessary in apoptosis, either via a caspase-8 or caspase-9 pathway.

Apoptosis-inducing factor (AIF) is a 57 kDa protein that resides mainly within the space between the inner and outer mitochondrial membrane. Upon loss of mitochondrial membrane integrity, AIF is released from the mitochondria to induce nuclear condensation and large-scale DNA fragmentation (15). This leads to cell death without the participation of caspases; hence, AIF is a key player in eliciting caspase-independent apoptosis in the cells.

The aim of this study was to examine whether ursolic acid induced lung cancer cell apoptosis. Since in ursolic acid-induced H460 cell apoptosis the role of MMP members was unclear, this study characterized the effect of ursolic acid on the gene expression of MMP members. Furthermore, the localization of the glucocorticoid receptor, which has been reported to be an important factor for MMP expression, in ursolic acid-induced apoptosis was examined in the human lung non-small carcinoma H460 cells.

Materials and Methods

Materials. Antipain, aprotinin, dithiothreitol (DTT), EDTA, ethyleneglycol-bis-(β-aminoethyl ether)-N,N′,N′-tetraacetic acid (EGTA), leupeptin, pepstatin, phenylmethylsulfonyl fluoride (PMSF), Tris and ursolic acid (3β-hydroxy-urs-12-en-28-oic acid) were purchased from the Sigma Chemical Company (St. Louis, MO, USA); anti-rabbit IgG peroxidase-conjugated secondary antibody was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Antibodies to various proteins were obtained from the following sources: glucocorticoid receptor antibody was purchased from the Sigma Chemical Company; caspase-3 was purchased from the Sigma Chemical Company (St. Louis, MO, USA); anti-rabbit IgG peroxidase-conjugated secondary antibody was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Enhanced chemiluminescent (Renaissance) detection reagents were obtained from NEN Life Science Products (Boston, MA, USA).

Cell culture. The human lung non-small carcinoma cell line H460 was grown in monolayer culture in Dulbecco’s modified Eagle’s medium (Life Technologies, Rockville, MD, USA) containing 5% fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, Rockville, MD, USA) and 2 mM glutamine (Merck, Darmstadt, Germany) at 37°C in a humidified atmosphere comprised of 95% air and 5% CO2. When H460 cells were treated with ursolic acid, the culture medium containing 1% fetal bovine serum was used. All data presented in this report are from at least three independent experiments showing the same pattern of expression.

Cell viability assay. Cells were seeded at a density of 5x10^4 cells/well onto 12-well plates (Falcon, Franklin Lakes, NJ, USA) 48 h before treatment. Drug was added to the medium at 3, 10 and 30 μM. The control cultures were treated with 0.1% DMSO (dimethylsulfoxide; Merck). After incubation, the cells were washed with PBS (phosphate-buffered saline). The number of viable cells was determined by staining the cell population with Trypan blue (Sigma). One part of 0.2% Trypan blue dissolved in PBS was added to one part of the cell suspension, and the number of unstained (viable) cells was counted under a microscope.

Migration assay. The migratory activity of H460 cells was assessed using a wounded migration assay. Cells were seeded at a density of 5x10^4 cells/well onto 12-well plates (Falcon) 48 h before treatment. Monolayers in 12-well plates were wounded with pipette tips following two perpendicular lines, giving rise to two acellular 1-mm-wide lanes per well. After washing, cells were supplied with 1 ml complete medium in the absence (control) or presence of different concentrations of ursolic acid. Wounded areas were photographed. After 24 h incubation, plates were observed under a microscope and photographs were taken of the same areas.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNAs were isolated from control or ursolic acid-treated H460 cells with an RNeasy Mini kit (QIAGEN, USA) according to the manufacturer’s instructions. RNA concentration was quantified using a spectrophotometer at a wavelength of 260 nm. cDNA was prepared by reverse transcription of 1.5 μg total RNA. Gene transcripts were determined by RT-PCR using an RNA PCR kit (Invitrogen Life Technologies, USA). The primers of investigated genes are shown in Table I. The amplification was performed with one denaturing cycle at 95°C for 5 min, then 30 cycles at 95°C for 1 min, at 55°C for 1 min, at 72°C for 1 min, and one final extension at 72°C for 10 min. RT-PCR products were separated by electrophoresis on 2% agarose gel and visualized by ethidium bromide staining.
Cell cycle analysis. Briefly, 2x10^6 cells were trypsinized, washed twice with PBS and fixed in 80% ethanol. Fixed cells were washed with PBS, incubated with 100 μg/ml RNase A for 30 min at 37°C, stained with propidium iodide (50 μg/ml) and analyzed on a FACScan flow cytometer (BD Biosciences, Palo Alto, CA, USA). The repeated average of the percentages of each phase in the cell cycle was representative of three independent experiments.

Immunostaining. Cells were seeded at a density of 5x10^4 cells onto glass plates 48 h before treatment. Cells were fixed with formaldehyde, permeabilized with 1% Triton X-100, blocked with 2.5% bovine serum albumin, incubated with glucocorticoid receptor antibody, and then stained with fluorescein-conjugated anti-rabbit IgG antibody. Thereafter, cells were co-stained with 1 μg/ml DAPI (Sigma), a fluorescent dye specific for DNA, for 5 min. After three washings in Tris-buffered saline (10 mM Tris, 150 mM NaCl (Sigma)) with 0.05% Tween 20 (TBST; Merck), the specimens were mounted in glycerin and observed under confocal spectral microscopy (Leica TCS SP2, Leica Microsystems, Germany).

Protein preparation. Adherent and floating cells were collected at the indicated time intervals (4, 8, 16 and 24 h) and washed twice in ice-cold PBS. Cell pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin and 5 μg/ml antipain) for 30 min at 4°C. Lysates were clarified by centrifugation at 13,000 rpm for 30 min at 4°C and the resulting supernatant was collected, aliquoted (50 μg/tube) and stored at –80°C until assayed. The protein concentrations were estimated using the Bradford method (16).

Western blot analysis. Samples were separated by various appropriate concentrations (11 and 13%) of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad, Hercules, CA, USA). The SDS-separated proteins were equilibrated in transfer buffer (Tris-HCl 50 mM, pH 9.0-9.4, glycine 40 mM, 0.375% SDS (Bio-Rad), 20% methanol (Merck)) and electrotransferred to Immobilon-P Transfer Membranes (Millipore Corporation, Bedford, MA, USA). The blot was blocked with a solution containing 5% non-fat dry milk in Tris-buffered saline (Tris-HCl 10 mM, NaCl 150 mM (Sigma)) with 0.05% Tween 20 (TBST; Merck) for 1 h, washed and incubated with antibodies to β-actin (1:5000 (Sigma)), the detection of β-actin was used as an internal control in all of the data of Western blotting analysis, AIF (1:1,000) and caspase-3 (1:1,000). Secondary antibody consisted of a 1:20,000 dilution of HRP-conjugated goat anti-rabbit IgG. The enhanced chemiluminescent (NEL Life Science Products) detection system was used for immunoblot protein detection.

4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) staining. Cells were seeded at a density of 5x10^4 cells/well onto 12-well plates 48 h before treatment. H460 cells were cultured for 24 h in 1% serum medium with vehicle alone (0.1% DMSO) or ursolic acid (3 or 10 μM). After treatment, cells were fixed with 3.7% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 and stained with 1 μg/ml DAPI for 5 min at 37°C. The cells were then washed with PBS and examined under fluorescence microscopy (Olympus IX 70, Olympus Optical Co., Germany).

Results

The effect of ursolic acid on cell proliferation in H460 cells. The present study evaluated the effects of ursolic acid on cell proliferation of H460 cells using Trypan blue dye exclusion. The data are presented as proportional viability (%) comparing the treated group with the untreated cells, the viability of which was assumed to be 100%. Twenty-four hours of continuous exposure to various concentrations of ursolic acid (3, 10 and 30 μM) on H460 cells resulted in dose-dependent decreases in cell number relative to control cultures (Figure 1). The IC_{50} (inhibitory concentration 50%) of ursolic acid was about 10 μM. Therefore, 10 μM ursolic acid was chosen for further experiments.

The effect of ursolic acid on the migration potential of H460 cells. Migration potential might be linked to cancer invasion. The previous observations reported that ursolic acid had anti-invasive activity in the human fibrosarcoma cells. In order to investigate whether the effect of ursolic acid on anti-invasive activity in H460 cells, the wounded migration assay was used. Ursolic acid seemed to produce a dose-dependent inhibitory effect, as observed 24 h after wounding (Figure 2).

The effect of ursolic acid on the gene expression of MMP family members of H460 cells. In order to further demonstrate whether the anti-invasive activity of ursolic acid is linked to MMP family members, RT-PCR techniques were used in this study. Soluble MMPs are involved in degradation of...
collagens, laminins and fibronectin and are produced by cancer cells, therefore, the present study investigated the gene expression of soluble MMPs, such as MMP-1, -2, -3, -8, -9, -10 and -13, after treatment with ursolic acid in H460 cells. After H460 cells were treated with 10 μM ursolic acid for 24 h, there was a significant increase in the gene expression of MMP-1, -2, -3, -9 and -10 in H460 cells (Figure 3). MMP-13 gene expression was not found in H460 cells even when double the concentration of cDNA was used in this study (data not shown). A very faint PCR product of MMP-8 was observed (data not shown). Due to ursolic acid-induced up-regulation of MMP family member gene expression, we hypothesized that ursolic acid induced H460 cell death or inhibited cell proliferation, but did not inhibit the invasion of cells, in cell exposed to 10 μM ursolic acid for 24 h.

**Ursolic acid-induced H460 cells S-phase arrest.** To further investigate ursolic acid-induced anti-proliferation of H460 cells, flow cytometric analysis was performed on cells treated with 10 μM ursolic acid for 6, 12, 18 and 24 h. After

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**Figure 2.** Effect of ursolic acid on the migration of H460 cells as revealed by the wound assay. H460 cells were wounded as described in Materials and Methods section. After washing, fresh culture medium, containing 0.1% DMSO (control) or various concentrations of ursolic acid, was added. Photographs were taken at time zero (A) and after 24 h of incubation in the absence (B) or the presence (C-F) of ursolic acid. Concentrations of ursolic acid: 1 μM (C), 10 μM (D), 20 μM (E) and 30 μM (F). Results are representative of three independent experiments.
fixation and permeabilization, cells were incubated with propidium iodide and the cell cycle analyzed. Cell cycle analysis revealed an S-phase arrest of cell cycle after treatment with ursolic acid for 18 h. As shown in Table II, significant S-phase arrest was indicated by decreased proportions of cells in G2/M-phase.

Studies on apoptotic events. In order to investigate whether the induction of apoptosis by ursolic acid in H460 cells was responsible for inhibited proliferation, the expression of apoptosis-inducing factor and caspase-3 proteins were determined by Western blotting analysis. The proform of caspase-3 decreased after treatment with 10 μM ursolic acid for 8 h (Figure 4). After H460 cells were treated with 10 μM ursolic acid for the indicated time intervals, there were no changes in the expression of AIF protein (Figure 4). This study further elucidates whether ursolic acid also induce both chromatin condensation and DNA fragmentation in H460 cells. Treatment with ursolic acid resulted in changes in nuclear morphology, as evidenced by staining with DAPI, a DNA binding dye. Condensation and fragmentation was seen in cells at 24 h after 10 μM ursolic acid treatment (Figure 5).

The phenotypic characteristics of ursolic acid-treated H460 cells were also evaluated by microscopic inspection of overall morphology. Apoptotic bodies were observed after H460 cells were treated with 10 μM ursolic acid for 24 h (Figure 6).

Based on above data, the appearance of apoptotic events, such as the activation of caspase-3, DNA condensation and formation of apoptotic bodies, indicated that ursolic acid-induced H460 cell death was indicative of typical apoptosis.

### Table II. Ursolic acid-induced S-phase arrest in flow cytometry assay.

<table>
<thead>
<tr>
<th>Treatment time</th>
<th>Treatment</th>
<th>% of cells in phase</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>G2/G1</td>
</tr>
<tr>
<td>12 h DMSO</td>
<td>57±4</td>
<td>24±4</td>
</tr>
<tr>
<td>Ursolic acid</td>
<td>56±3</td>
<td>23±3</td>
</tr>
<tr>
<td>18 h DMSO</td>
<td>57±1</td>
<td>26±4</td>
</tr>
<tr>
<td>Ursolic acid</td>
<td>54±3</td>
<td>39±3</td>
</tr>
<tr>
<td>24 h DMSO</td>
<td>52±4</td>
<td>25±3</td>
</tr>
<tr>
<td>Ursolic acid</td>
<td>50±2</td>
<td>35±1</td>
</tr>
</tbody>
</table>

Cells were treated with vehicle alone or 10 μM ursolic acid in the presence of 1% serum for the time periods shown. After treatment, cells were harvested and subjected to flow cytometric analysis. The percentages of cell at each phase in the cell cycle were representative of three independent experiments.
The effects of ursolic acid on immunofluorescence localization of glucocorticoid receptor (GR) in H460 cells. The immunoreactivity of glucocorticoid receptor appeared as diffuse staining throughout control cells (Figure 7). A marked cytosolic localization of the GR was observed after treatment with 10 μM ursolic acid for 24 h (Figure 7). At this time, the dotted staining and bright green fluorescence of GR was most intense in the perinucleus (Figure 7).

Discussion

Ursolic acid has numerous pharmacological activities including anti-inflammatory, anti-cancer and hepatoprotective effects (17-19). It has been suggested that ursolic acid inhibits proliferation and stimulates apoptosis in colon cancer HT29 cells (19). Ursolic acid also has been demonstrated to inhibit the invasion of HT 1080 human fibrosarcoma cells by reducing the expression of MMP-9 (4). In the present study, the obvious effect of anti-proliferation or anti-migration of ursolic acid on human lung cancer cells was observed. Therefore, we focused on the effect of ursolic acid on anti-invasive activity.

MMPs play an important role in wound healing, angiogenesis, embryogenesis and in pathological processes, such as tumor invasion and metastasis. Of these, 23 MMPs have been found to be expressed in human cells or tissues. MMPs can be divided into two distinct groups, the secreted MMPs and the membrane-type MMPs. Soluble MMPs involved in the degradation of collagens, laminins and fibronectin are produced by cancer cells, raising the possibility that they might contribute to their invasion across basement membranes and interstitial tissues. The proteolytic degradation of the ECM by tumor cells requires the action of highly specialized MMPs during tumor invasion (20-22). This study investigated whether the anti-invasive activity of ursolic acid is linked to MMP family members. Therefore, the expression of soluble MMPs, such as MMP-1, -2, -3, -8, -9, -10 and -13, was investigated after treatment with ursolic acid in H460 cells, using RT-PCR techniques. With the exception of MMP-8 and -13, there was a significant increase in the expression of MMP-1, -2, -3, -9 and -10 after H460 cell treatment with 10 μM ursolic acid for 24 h. This result is not consistent with previous observations in which ursolic acid had anti-invasive activity in human fibrosarcoma cell with a reduction of the expression of MMP-9 (4, 23). It is noteworthy that remodelling of the cell matrix, and cell-to-cell contact is required not only in the invasion of tumor cells, but also in cell apoptosis (12). The regulation of MMP on migration, proliferation and death has been suggested in vascular smooth muscle cells (12). Cárdenas et al. have also demonstrated that ursolic acid was able to inhibit key steps of angiogenesis, including endothelial cell proliferation, migration and differentiation, and it seemed to stimulate the activity of MMP-2 at the same time (24). Furthermore, MMPs, such as MMP-1, -2, -8, -9 and -13 to -17, have been demonstrated to modulate apoptosis by cleaving death ligands (e.g., TNF-α and
Based on the above reasons, it was hypothesized that treatment with 10 μM ursolic acid for 24 h could inhibit H460 cell proliferation and induce cell apoptosis. Up-regulation of MMPs is involved in ursolic acid-induced H460 cell apoptosis.

It was found that ursolic acid caused a dose-dependent growth inhibition in H460 cells. However, the inhibition of cell growth observed in ursolic acid-treated cells was due to induction of S-phase cell cycle arrest. Flow cytometry results revealed a reduction in the G2/M-phase of the cell division cycle. Based on the above results, H460 cells failing to progress to mitosis may be destined to apoptosis by ursolic acid. Apoptosis is a major form of cell death. The present study demonstrated that ursolic acid induced apoptosis, as revealed by the appearance of typical fragmented and condensed nuclei in apoptotic cells, which have been widely used as biochemical markers of apoptosis, in H460 cells.

It is widely accepted that there are two principal pathways of apoptosis – caspase-dependent and -independent. Among the members of the caspase family, caspase-3 is required for many of the nuclear changes associated with apoptosis, including DNA fragmentation and chromatin condensation. The expression of proform of caspase-3 was significantly decreased after treatment with ursolic acid in this study. Ursolic acid had no effect on the expression of AIF protein in the present study. It was further demonstrated that ursolic

Figure 6. Changes in H460 cell morphology during ursolic acid-induced apoptotic cell death. Shown are phase-contrast views of H460 cells cultured for 4, 12 and 24 h with control (A, C, E respectively) or in the presence of 10 μM ursolic acid (B, D, F respectively).
Ursolic acid induced both chromatin condensation and DNA fragmentation in H460 cells based on DAPI staining. From the above results, in this study ursolic acid was shown to be inducing caspase-dependent apoptosis due to its capability to damage DNA, via activation of caspase-3.

Glucocorticoid hormones have been reported to be potent inhibitors of MMPs; their expression can be induced by activator protein 1 (AP-1) stimulated by phorbol esters (27, 28). During the process of the MMPs inhibition, glucocorticoid hormone binding with its receptor and translocation to the nucleus is necessary. Since the chemical structure of ursolic acid is very similar to that of glucocorticoid hormone, the present study investigated whether the changes of the localization of the glucocorticoid receptor was involved in ursolic acid-induced H460 cell apoptosis. The results showed a marked cytosolic localization of the glucocorticoid receptor after treatment with 10 μM ursolic acid for 24 h. At this time, the dotted staining and bright green fluorescence of the glucocorticoid receptor was most intense in the perinucleus. The translocation of the glucocorticoid receptor from the cytosol to nucleus was not observed in this study. Cha et al. reported that ursolic acid-induced down-regulation of the MMP-9 gene is mediated through the nuclear translocation of the glucocorticoid receptor (4). Therefore, the localization of the glucocorticoid receptor in the cytosol might be an important factor in inducing MMP up-regulation during ursolic acid-induced H460 cell apoptosis, as found in this study. The relationship between the activation of the glucocorticoid receptor and the expression of MMP will be investigated in the future.

**Conclusion**

It was demonstrated that ursolic acid induced a significant increase in the gene expression of MMP-1, -2, -3, -9 and -10 after treatment with 10 μM ursolic acid for 24 h in H460 cells. Under these experimental conditions, ursolic acid induced typical apoptosis in H460 cells. Ursolic acid-induced apoptosis was characterized by activation of caspase-3, nuclear morphological changes and DNA fragmentation. Our study suggests that the localization of the glucocorticoid receptor in cytosol may be an important factor in inducing MMP up-regulation during ursolic acid-induced H460 cell apoptosis.
References