Role of human longevity assurance gene 1 and C_{18}-ceramide in chemotherapy-induced cell death in human head and neck squamous cell carcinomas

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Abstract

In this study, quantitative isobologram studies showed that treatment with gemcitabine and doxorubicin, known inducers of ceramide generation, in combination, supra-additively inhibited the growth of human UM-SCC-22A cells *in situ*. Then, possible involvement of the human homologue of yeast longevity assurance gene 1 (LASS1)/C_{18}-ceramide in chemotherapy-induced cell death in these cells was examined. Gemcitabine/doxorubicin combination treatment resulted in the elevation of mRNA and protein levels of LASS1 and not LASS2-6, which was consistent with a 3.5-fold increase in the endogenous (dihydro)ceramide synthase activity of LASS1 for the generation of C_{18}-ceramide. Importantly, the overexpression of LASS1 (both human and mouse homologues) enhanced the growth-inhibitory effects of gemcitabine/doxorubicin with a concomitant induction of caspase-3 activation. In reciprocal experiments, partial inhibition of human LASS1 expression using small interfering RNA (siRNA) prevented cell death by about 50% in response to gemcitabine/doxorubicin. In addition, LASS1, and not LASS5, siRNA modulated the activation of caspase-3 and caspase-9, but not caspase-8, in response to this combination. Treatment with gemcitabine/doxorubicin in combination also resulted in a significant suppression of the head and neck squamous cell carcinoma (HNSCC) tumor growth in severe combined immunodeficiency mice bearing the UM-SCC-22A xenografts. More interestingly, analysis of endogenous ceramide levels in these tumors by liquid chromatography/mass spectroscopy showed that only the levels of C_{18}-ceramide, the main product of LASS1, were elevated significantly (about 7-fold) in response to gemcitabine/doxorubicin when compared with controls. In conclusion, these data suggest an important role for LASS1/C_{18}-ceramide in gemcitabine/doxorubicin-induced cell death via the activation of caspase-9/3 in HNSCC. [Mol Cancer Ther 2007;6(2):712–22]

Introduction

Squamous cell carcinoma of the head and neck (HNSCC) remains one of the leading causes for cancer-related deaths in the United States. Five-year survival rate for patients with grades III and IV HNSCC is still about 50%, and it did not improve for decades. (1, 2) Therefore, the development of novel therapeutic strategies for improved survival outcome of HNSCC is needed.

The bioactive sphingolipid ceramide, which is emerging as a tumor suppressor lipid, has been shown to regulate the activity of various biochemical and molecular targets involved in antiproliferative responses (3, 4). It is known that increased ceramide generation in response to various stress stimuli including radiation and chemotherapeutic agents, or treatment with exogenous ceramides, can result in the inhibition of growth and/or induction of cell death in various cancer cells *in situ* (5–8). Indeed, the role for exogenous ceramide against HNSCC growth was shown previously, which showed that the combination of paclitaxel and C_6-ceramide synergistically decreased the growth of the human Tu138 HNSCC cells (9). Similarly, in an independent study, the inhibition of growth in various HNSCC cell lines, but not in normal keratinocytes, by treatment with a recently developed L-threo-C_6-pyridinium ceramide with high bioavailability and bioactivity was reported (10).

Roles for endogenous ceramide in chemotherapy-induced cell death have also been well documented (8). It has been shown previously that endogenous ceramide can be generated by various mechanisms, including *de novo* synthesis of ceramide, or activation of sphingomyelinases (11–13). One of the key enzymes of the *de novo* pathway is

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(dihydro)ceramide synthase, and it has been shown that in *Saccharomyces cerevisiae*, longevity assurance gene (LAG) members (LAG1p and Lac1p) play a role in the regulation of life span (14) and are also required for ceramide synthase activity (15, 16). Recent studies also revealed that one of the mouse homologues of these proteins, known as upstream of growth and differentiation factor 1 (mUOG1 or mouse LASS1) specifically regulates the synthesis of stearoyl (C_{18})-containing sphingolipids, including C_{18}-ceramide (17, 18). The human homologue of the LAG1 was also identified (named as LASS1; ref. 19). Recently, defects in the generation of C_{18}-ceramide were implicated in the pathogenesis and/or progression of SCC, but not in non-SCC, of the head and neck tumors (20).

In this study, the role for LASS1 via the generation of C_{18}-ceramide in drug-induced cell death in HNSCC cells was examined. Increased efficacy of the combination of gemcitabine and doxorubicin, which are known inducers of ceramide generation, against HNSCC has been shown both *in situ* and *in vivo*. More importantly, the data presented here show that LASS1/C_{18}-ceramide pathway plays important roles in the regulation of gemcitabine/doxorubicin-induced caspase activation and cell death in HNSCC cells.

Materials and Methods

Cell Lines and Culture Conditions

Human head and neck cancer cell lines UM-SCC-22A, UM-SCC-14A, and UM-SCC-1 cells were obtained from Dr. Thomas Carey (Department of Otolaryngology/Head and Neck Surgery, University of Michigan). Cells were grown in DMEM containing 10% FCS and 1% penicillin/streptomycin at 37°C in 5% CO_2 as described previously (10).

Detection of Growth Inhibition by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide and Isobologram Studies

The concentrations of agents that inhibited cell growth by 50% (IC_{50}) were determined from cell growth plots obtained by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as described (21). Triplicate wells were used for each treatment. The final concentration of DMSO (solvent for gemcitabine and doxorubicin) in the growth medium was <0.1% (v/v), which has no effect on cell growth and survival. The supra-additivity of gemcitabine and doxorubicin in combination was examined by isobologram studies done as described previously (21).

Quantitative Real-Time PCR, Conventional RT-PCR, and Western Blotting

One microgram of total RNA, isolated using an RNA isolation kit (Qiagen, Valencia, CA), was used in reverse transcription reactions as described (22). The resulting total cDNA was then used in the quantitative real-time PCR (Q-RT-PCR) to measure the mRNA levels using TaqMan gene expression kit (Applied Biosystems, Foster City, CA) with ABI 7300 Q-PCR system. The mRNA levels of β-actin and rRNA were used as internal controls. Conventional RT-PCR primers and conditions are as follows: LASS1 (forward), 5′-CTATACATGGACACCTGGCGCAA-3′, (reverse) 5′-TCAGAA-GCGCTTGTCCTTCACCA-3; and LASS5 (forward) 5′-CCCTCGAGGGATG*GA-TTACAAGGATGACG-ACGATAAG*-ATGGCGACAGCAGCGCAGGGA-3′, and (reverse), 5′-CGGAATTCCGTTACTCTTCAG-CCCAG-TAGCT-3′ (annealing temperatures were 68°C and 55°C, respectively).

The protein levels of human LASS1 and LASS6 were detected by Western blotting using mouse polyclonal antibodies (Abnova) as described previously (22).

Plasmids and Small Interfering RNAs

Transfections of cells were done using Effectene Transfection Kit (Qiagen) as described previously (8). The small interfering RNAs (siRNA) against human LASS1 were synthesized and High Performance Purity (HPP) purified by Qiagen Biotechnology, and transfections of cells with siRNA molecules were done using OligofectAMINE Transfection Kit (Invitrogen, Carlsbad, CA) as described by the manufacturer. The target sequences for siRNAs were as follows: LASS1, AAGGTCCTGTATGCCACCAGT; hLASS5, AAA-CCCTGTGCACTCTGTATT; and nontargeting control, AATTCTCCGAACGTG-TCACGT.

Detection of (Dihydro)ceramide Synthase Activity *In vitro*

An *in vitro* enzyme activity for CoA-dependent (dihydro)ceramide synthase was measured using microsomal preparations of UM-SCC-22A cells for the incorporation of stearoyl- or palmitoyl CoA into [^{3}H]dihydro-sphingosine for the generation of C_{18}- or C_{16}-ceramides, respectively, as described previously (17).

Measurement of Endogenous (Dihydro)ceramide Synthase Activity, and Absolute Ceramide Levels Using High-Performance Liquid Chromatography/Mass Spectroscopy

The endogenous activity of (dihydro)ceramide synthase was measured by monitoring the generation of 17C-(dihydro)ceramides in cells after pulsing cells with 5 μM 17C-dihydro-sphingosine, an unnatural form of sphingosine containing 17-carbon backbone as opposed to its natural form with 18-carbons, for 4 h by liquid chromatography/mass spectroscopy (LC/MS), as described previously (23). Similarly, cellular levels of endogenous ceramides were measured by LC/MS as described previously (24). Ceramide levels were normalized to total protein amounts or total inorganic phosphate levels.

Detection of Caspase Activation by Fluorometry, Western Blotting, and Immunofluorescence

The activation of caspase-3 was measured using Caspase3 activity assay kit (R&D Systems, Minneapolis, MN) by fluorometry as described by the manufacturer. The levels of active caspase-3, caspase-8, or caspase-9 were also measured by Western blotting using antibodies against caspase-3 (Trevigen, Gaithersburg, MD), caspase-8, and caspase-9 (Cell Signaling Technology, Danvers, MA) as described previously (22). The active caspase-3 levels were also measured by immunofluorescence using active caspase-3–specific antibody as described by the manufacturer.

In brief, pIRES/GFP- or pIRES/GFP/LASS1-transfected cells were fixed in 4% paraformaldehyde at 25°C for 10 min and permeabilized in 0.1% Triton X-100 and 4% paraformaldehyde at 25°C for 10 min. After blocking in 1% bovine serum albumin for 1 h, cells were incubated with antiactive caspase-3 antibody (Trevigen) for 1 h in 1% bovine serum albumin. Rhodamine-conjugated donkey anti-rabbit immunoglobulin G (Jackson Immuno Research, West Grove, PA) was used as a secondary antibody. Then, the active form of caspase-3 was visualized in green fluorescent protein (GFP)–positive cells by immunofluorescent microscopy.

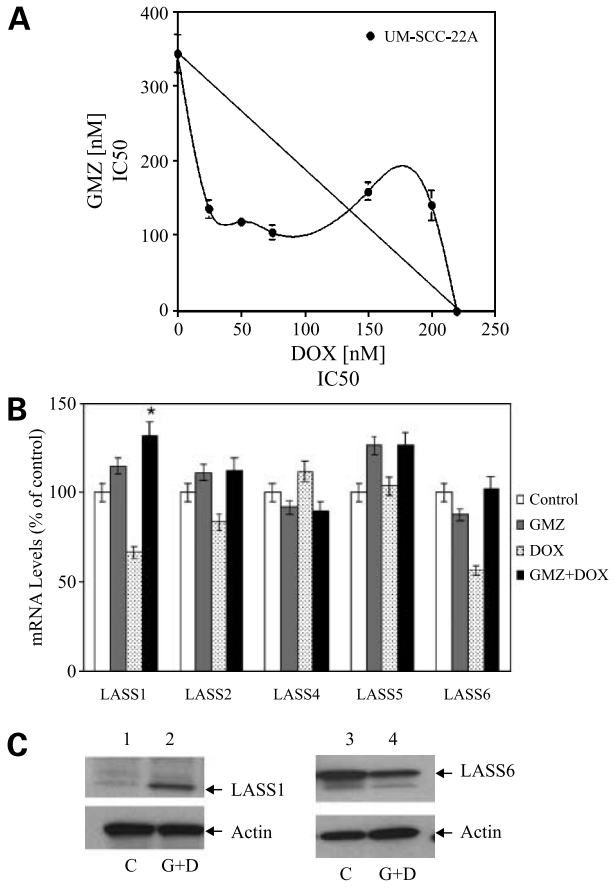

Figure 1. Improved growth inhibitory effects of gemcitabine, in combination with doxorubicin, on UM-SCC-22A cells *in situ* and change of expression levels of LASS genes in response to gemcitabine/doxorubicin treatment. **A,** the supra-additive interaction of gemcitabine in combination with doxorubicin in the inhibition of growth of UM-SCC-22A cells was determined by quantitative isobologram studies as described in Materials and Methods. The IC_{50} concentrations of gemcitabine in the presence of increasing concentrations of doxorubicin at 48 h were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays, and the data were plotted in isobolograms. A straight line joining the points on the *X*- and *Y*-axes represent the IC_{50} concentrations of gemcitabine and doxorubicin alone. The results represent at least three independent experiments, which were done in triplicates. *Bars,* SD. **B,** UM-SCC-22A cells were treated with gemcitabine (150 nmol/L), doxorubicin (75 nmol/L), or gemcitabine and doxorubicin (150 and 75 nmol/L, respectively) in combination for 48 h. After the treatments, LASS1, LASS2, LASS4, LASS5, and LASS6 mRNA expression levels were determined by Q-PCR as described in Materials and Methods. Statistical analysis was done as described in Materials and Methods. *, $P < 0.05$, statistically significant. **C,** levels of LASS1 and LASS6 proteins were determined by Western blotting upon treatment with gemcitabine (150 nmol/L), doxorubicin (75 nmol/L), or gemcitabine and doxorubicin (150 and 75 nmol/L, respectively) in combination for 48 h. Actin levels are used as loading controls.

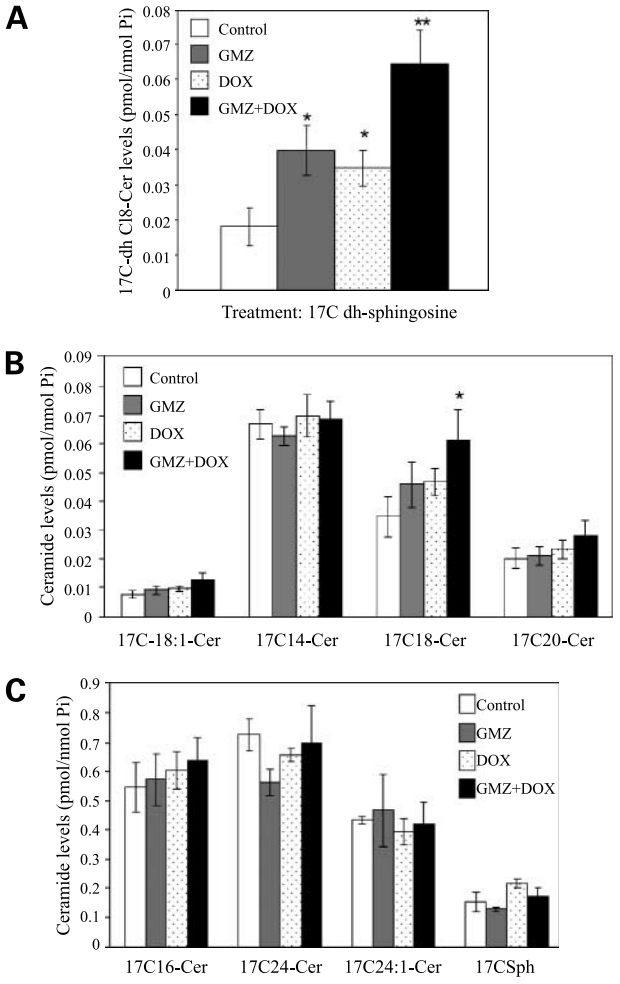

Figure 2. Role of gemcitabine/doxorubicin on the conversion of 17C-dihydrosphingosine to 17C-ceramides. **A,** endogenous conversion of 17C-dihydrosphingosine to 17C-dihydro-C_{18}-ceramide in response to treatments with gemcitabine, doxorubicin, or gemcitabine and doxorubicin in combination was measured. UM-SCC-22A cells were treated with gemcitabine (150 nmol/L), doxorubicin (75 nmol/L), or gemcitabine and doxorubicin (150 and 75 nmol/L, respectively) in combination for 44 h and pulsed with 17C-dihydrosphingosine (5 μmol/L) for an additional 4 h. Then, the generation of 17C(dihydro)-C_{18}-ceramide was measured by LC/MS. The levels of ceramides were normalized to total inorganic phosphates. The data are representative of two independent experiments done in duplicates. *Bars,* SD. The generation of (**B**) $17C_{18:1}$-, $17C_{14}$-, $17C_{18}$-, and $17C_{20}$-ceramides or (**C**) $17C_{16}$-, $17C_{24}$-, and $17C_{24:1}$-ceramides or 17C-sphingosine in response to gemcitabine, doxorubicin, or gemcitabine/doxorubicin in these cells were also measured by LC/MS. The results are representative of at least two independent experiments; *bars*, SD. Statistical analysis was done as described in Materials and Methods. *, $P < 0.05$, statistically significant.

Determination of the Effects of Gemcitabine/Doxorubicin on HNSCC Tumor Growth *In vivo*

UM-SCC-22A cell xenografts were generated by the injection of 1×10^6 cells in the flanks of the severe

combined immunodeficient (SCID) mice. After tumors were grown to at least 200 mm^3 (about 2 weeks after implantation), mice were treated i.p. without or with gemcitabine (40 mg/kg), doxorubicin (1 mg/kg), or gemcitabine and doxorubicin in combination (40 and 1 mg/kg, respectively) every 4 days for 32 days. At each treatment, tumors were measured, and tumor volumes were calculated as described (25).

Statistical Analysis

Statistical significance of the data from the xenografts was analyzed using Tukey's Student's test as described previously (26). Treatment of animals was done according to the approved procedures by the Institutional Animal Care and Use Committee at the Medical University of South Carolina.

Results

Inhibition of HNSCC Cell Growth in Response to the Combination of Gemcitabine and Doxorubicin Treatment *In situ*

The effective growth-inhibitory roles of gemcitabine and doxorubicin as single agents against HNSCC cell lines *in situ* have been shown previously (27, 28). Because these agents are known inducers of endogenous ceramide generation (11, 29), possibly by different mechanisms, we hypothesized that they might be supra-additive when used in combination against HNSCC cells. This was first examined using quantitative isobologram studies *in situ* (21). As seen in Fig. 1A, treatment with gemcitabine in the presence of increasing concentrations of doxorubicin for 48 h synergistically inhibited the growth of UM-SCC-22A cells. Specifically, the combination of doxorubicin at its sub-IC_{50} values (25-75 nM) with increasing concentrations of gemcitabine (75-150 nM) for 48 h shifted the IC_{50} values of gemcitabine in the isobologram to the left of the line plot joining the *Y*- and *X*-axes that represent the IC_{50} of gemcitabine, and increasing concentrations of doxorubicin, respectively, demonstrating supra-additivity. Interestingly, the combination of gemcitabine with higher concentrations (150-250 nM) of doxorubicin showed antagonism, shifting the IC_{50} values of gemcitabine in the isobologram to the right of the line plot (Fig. 1A).

The Effect of Gemcitabine/Doxorubicin on the Levels of LASS1 mRNA, Protein, and Its Dihydroceramide Synthase Activity in UM-SCC-22A Cells *In situ*

Because both gemcitabine and doxorubicin were shown to induce ceramide generation via the *de novo* pathway (11, 29), we examined the possible changes in the mRNA expression profiles of LASS genes, which are shown to be the bona fide ceramide synthases of the *de novo* pathway (30, 31) upon gemcitabine/doxorubicin treatment. To examine the effects of gemcitabine/doxorubicin (150 and 75 nmol/L, respectively), alone or in combination, on the mRNA levels of LASS1, UM-SCC-22A cells were treated

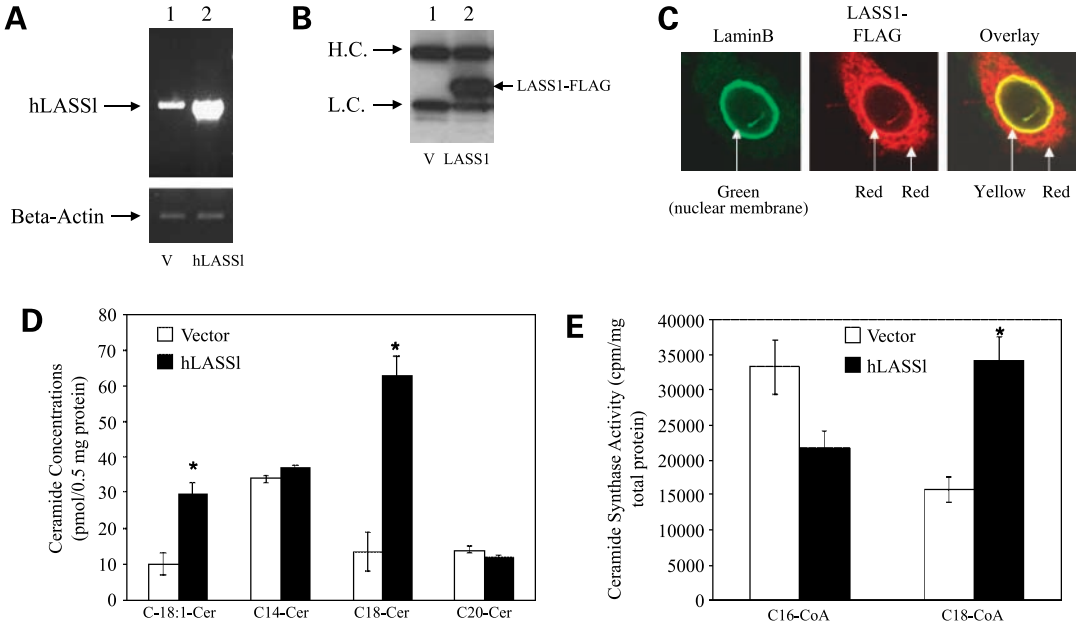

Figure 3. Induction of human LASS1 expression, which regulates ceramide synthase activity for the generation of C_{18}-ceramide levels. **A,** overexpression of LASS1 (*lane 2*) was confirmed by RT-PCR, and compared with that of controls (*lane 1*) as described in Materials and Methods. **B,** partial purification of the FLAG-tagged protein by anti-FLAG antibody-conjugated agarose beads followed by Western blotting using the antibody against FLAG epitope as described in Materials and Methods. Vector (*V*)-transfected cells were used as control. *L.C.* and *H.C.*, light-chain and heavy-chain peptides of the antibody used in immunoprecipitation. **C,** cellular localization of LASS1 was determined by immunofluorescence confocal microscopy using anti-FLAG and anti-LaminB antibodies as described in Materials and Methods. **D,** UM-SCC-22A cells were transfected with LASS1 for 48 h, and endogenous ceramide levels were measured by LC/MS. The ceramide levels were normalized to 0.5 mg total proteins. At least three experiments were done in duplicates; *bars,* SD. **E,** UM-SCC-22A cells were transfected with LASS1 for 48 h, and total microsomes were used to determine C_{16}- and C_{18}-ceramide synthase activity *in vitro* as described in Materials and Methods. Statistical analysis was done as described in Materials and Methods. *, $P < 0.05$, statistically significant.

with these drugs for 48 h, and the levels of LASS1 mRNA were quantitatively measured by real-time PCR as described. Treatment with gemcitabine/doxorubicin in combination increased the mRNA levels of LASS1 by about 30% when compared with untreated controls (Fig. 1B). There was no detectable change in the mRNA levels of hLASS2-6 (Fig. 1B) in response to these drugs in these cells. When protein levels of human LASS1 were analyzed, the data showed that treatment with gemcitabine/doxorubicin in combination resulted in a significant increase in the protein levels of LASS1 in these cells when compared with untreated controls (Fig. 1C, *lanes 2* and *1*, respectively, whereas the expression of human LASS6 was down-regulated in response to these drugs (Fig. 1C, *lanes 4* and *3*, respectively). These data suggest that the down-regulation of LASS6 in response to gemcitabine/doxorubicin is controlled at the posttranscriptional level by an unidentified mechanism.

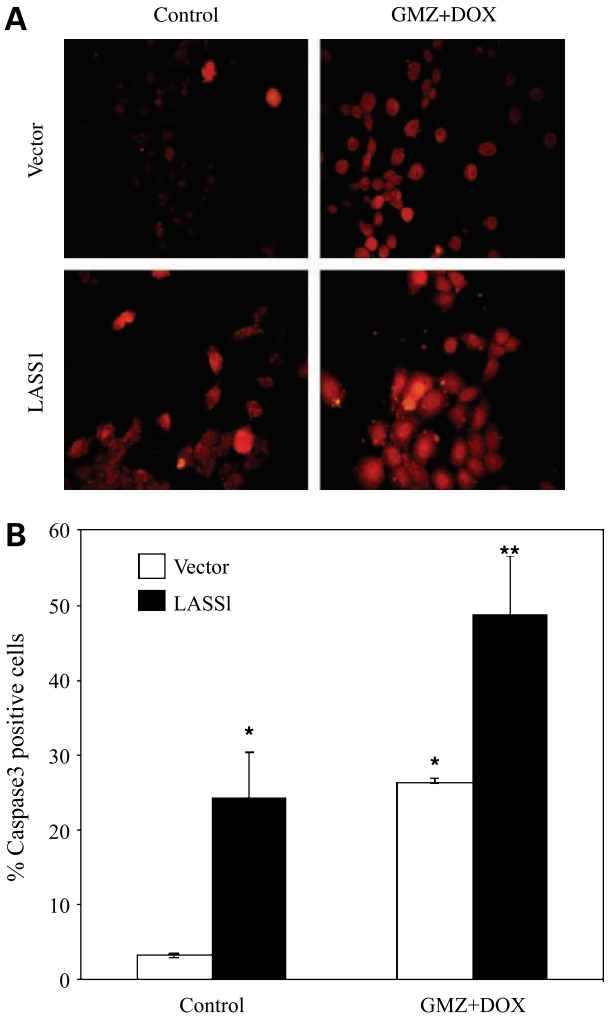

Figure 4. Role of LASS1 in caspase-3 activation in UM-SCC-22A cells. **A**, UM-SCC-22A cells were transfected with either pIRES/GFP or pIRES/LASS1/GFP for 36 h and then treated with gemcitabine/doxorubicin for an additional 48 h. After gemcitabine/doxorubicin treatments, the cells were stained with anticleaved caspase-3 antibody, and cells were visualized with immunofluorescence microscopy as described in Materials and Methods. **B**, quantitative representation of cleaved caspase-3 in GFP-positive cells was determined in experiments described in Fig. 4A. The cells in at least three different fields of view were counted for quantification. *Bars,* SD. Statistical analysis was done as described in Materials and Methods. *, $P < 0.05$; and **, $P < 0.01$, statistically significant.

Next, the effects of gemcitabine/doxorubicin, alone or in combination, on the dihydroceramide synthase activity (both *in vitro* and endogenous) was determined as described in Materials and Methods. Interestingly, there was no significant effect of these drugs on the *in vitro* enzyme activity, which was measured using microsomal preparations of drug-treated or untreated cells for the incorporation of stearoyl- or palmitoyl-CoA to the ^{3}H-(dihydro)sphingosine for the generation of C_{18}- or C_{16}-ceramides (data not shown), measured as described previously (20). Then, to determine the effects of these drugs on the endogenous enzyme activity, after treatment with gemcitabine/doxorubicin for 44 h, cells were pulsed with 17C-(dihydro)sphingosine (5 μM for an additional 4 h), and its conversion to C_{17}-(dihydro)ceramides in these cells was measured by LC/MS as described previously (23, 32). The data showed that gemcitabine/doxorubicin in combination significantly increased endogenous (dihydro)-ceramide synthase activity for the conversion of 17C-(dihydro)-sphingosine to 17C-dihydro-C_{18}-ceramide about 3.5-fold, whereas single-agent treatments with gemcitabine or doxorubicin increased the *in situ* enzyme activity by about 2- and 1.6-fold, respectively, when compared with untreated controls (Fig. 2A). Importantly, gemcitabine/doxorubicin combination also increased the generation of 17C_{18}-ceramide by about 50%, whereas it did not have any significant effect on the generation of 17C_{14}-, 17C_{16}-, 17C_{20}-, or 17C_{24}-ceramides, compared with controls (Fig. 2B and C).

Therefore, these data show that treatment with gemcitabine/doxorubicin in combination increased the endogenous enzyme activity of LASS1 for the generation of C_{18}-ceramide in these cells.

The Role of LASS1 Expression in Gemcitabine/Doxorubicin-Induced Cell Death and Caspase Activation

To determine the roles of increased C_{18}-ceramide generation in gemcitabine/doxorubicin-induced cell death, UM-SCC-22A cells were transfected with expression vectors containing the full-length LASS1 cDNA with FLAG tag on its NH_2 terminus. Then, the effects of its overexpression, as confirmed by RT-PCR, Western blotting, and confocal microscopy (Fig. 3A-C), on the generation of C_{18}-ceramide were determined as compared with vector controls by LC/MS. As expected, LASS1 overexpression resulted in a significant increase specifically in C_{18}-ceramide levels (about 6.5-fold) when compared with controls, with no significant changes on the levels of other ceramides such as C_{14}- or C_{20}-ceramides (Fig. 3D). These data were further confirmed by detecting the effects of LASS1 overexpression on the *in vitro* activity of (dihydro)-ceramide synthase using microsomes of transfected cells as described in Materials and Methods. The results showed that overexpression of human LASS1 resulted in an

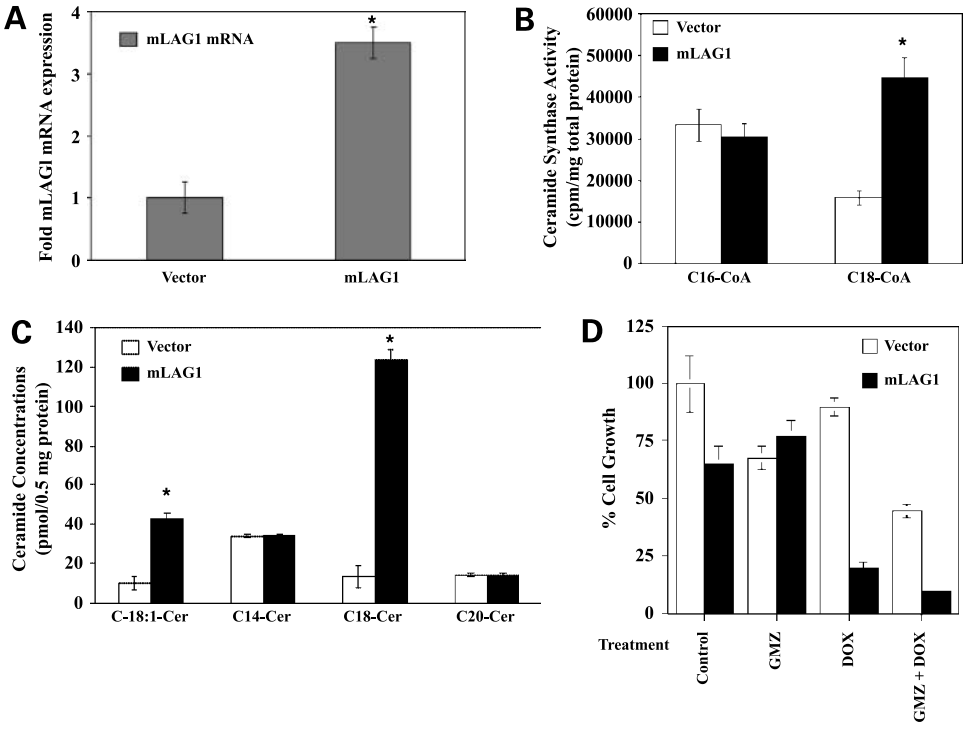

Figure 5. Roles of mLASS1/UOG1 expression on ceramide metabolism and gemcitabine/doxorubicin-induced growth inhibition in UM-SCC-22A cells. **A**, overexpression of mouse LASS1 in UM-SCC-22A cells were confirmed by Q-PCR as described in Materials and Methods. **B**, the effects of mouse LASS1 (mLASS1) on ceramide synthase activity for the generation of C_{18}-ceramide was confirmed by *in vitro* enzyme activity assay as described in Materials and Methods. **C**, the levels of endogenous ceramide in cells transfected with vector (*controls*) or with mLASS1 were measured by LC/MS. **D**, UM-SCC-22A cells were transfected with vector control or mLASS1 (for 36 h) before gemcitabine/doxorubicin treatments (48 h). After treatments, percent growth inhibition was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. The results represent at least three experiments, which were done in duplicates. *Bars,* SD. Statistical analysis was done as described in Materials and Methods. *, $P < 0.05$, statistically significant.

increased (dihydro)ceramide synthase activity about 2- to 3-fold when compared with controls *in vitro*, specifically for the generation of C_{18}-ceramide, and not C_{16}-ceramide (Fig. 3E), measured by the incorporation of stearoyl- or palmitoyl-CoA into [^{3}H]-labeled dihydrosphingosine, respectively. These results further confirmed the role of human LASS1 specifically in the generation of C_{18}-ceramide in these cells.

In addition, the role of increased generation of C_{18}-ceramide via LASS1 overexpression in gemcitabine/doxorubicin-induced caspase-3 activation was analyzed by immunofluorescence using an antibody that specifically detects the cleaved (active) form of caspase-3 (Fig. 4A). The overexpression of LASS1 in pIRES vector, which also contained GFP under the regulation of an independent promoter, allowed the identification of pIRES/LASS1-transfected cells (GFP positive, data not shown) using immunofluorescence (Fig. 4A). More importantly, the quantification of these data showed that treatment with gemcitabine/doxorubicin significantly increased the activation of caspase-3 in pIRES/GFP-transfected controls, whereas the effects of gemcitabine/doxorubicin combination on caspase activation were further enhanced (about 2-fold) in pIRES/LASS1/GFP-positive cells when compared with control transfectants (Fig. 4B).

Similar data were also observed when mouse LASS1/UOG1 (upstream of growth and differentiation factor 1; mLASS1) was overexpressed (about 3.5-fold increase in mRNA levels) in UM-SCC-22A cells, which resulted in increased *in vitro* enzyme activity (about 2.5-fold) and generation of C_{18}-ceramide (about 8-fold; Fig. 5A-C, respectively). More importantly, overexpression of mLASS1 further increased doxorubicin- or gemcitabine/doxorubicin-induced cell death significantly in these cells when compared with controls (Fig. 5D).

Thus, these data reveal that increased generation of C_{18}-ceramide by LASS1 overexpression enhances gemcitabine/doxorubicin-induced caspase-3 activation and cell death in these HNSCC cells.

Determining the Role of LASS1 in Gemcitabine/Doxorubicin-Induced Cell Death in UM-SCC-22A Cells

To determine the role of LASS1 in the growth-inhibitory activity of gemcitabine/doxorubicin more specifically, LASS1 expression was partially inhibited about 75% in the absence or presence of drugs as determined by Q-PCR (Fig. 6A) by using a siRNA in UM-SCC-22A cells. More importantly, LASS1 siRNA partially but significantly prevented the inhibition of cell growth by about 42% in response to treatment with gemcitabine/doxorubicin in these cells (Fig. 6B). These data revealed that LASS1 expression plays an important role for the growth-inhibitory activity of gemcitabine/doxorubicin against UM-SCC-22A cells.

To examine whether downstream mechanisms of drug-induced cell death via LASS1 involves caspase-dependent cell death, the activity of caspase-3 was measured in the presence of control versus LASS1 siRNA-transfected cells. The results showed that treatment with gemcitabine/doxorubicin in combination significantly induced (about 3.6-fold) the activation of caspase-3 in control cells, which was partially prevented about 45% by the partial inhibition of LASS1 expression using siRNA (Fig. 7A). Similarly, partial inhibition of LASS1 by siRNA in UM-SCC-14A and UM-SCC-1 cell lines partially blocked gemcitabine/

doxorubicin-induced caspase-3 activation (Fig. 7B). In addition, gemcitabine/doxorubicin treatment in combination resulted in a significant activation of caspase-9, and not caspase-8, in control cells (transfected with nontargeting siRNA) as determined by Western blotting (Fig. 7C, *lanes 1-4*, respectively). However, partial inhibition of LASS1 expression by siRNA prevented the cleavage/activation of procaspase-9 in response to these drugs (Fig. 7C, *lanes 5-8*). To examine the specificity of the role of LASS1 in chemotherapy-induced apoptosis, the expression of LASS4 and LASS5 were also inhibited by siRNA, confirmed by real-time PCR (data not shown), and their effects on caspase-9 activation were examined. The data showed that whereas LASS1 siRNA prevented gemcitabine/doxorubicin-induced caspase-9 activation, LASS4 or LASS5 siRNA did not have any significant effect (Fig. 7D), confirming the specificity of LASS1/C_{18}-ceramide signaling in this process.

Thus, these results show, for the first time, that LASS1 plays an important role in gemcitabine/doxorubicin-induced cell death via caspase-9/3–dependent mechanism in UM-SCC-22A cells.

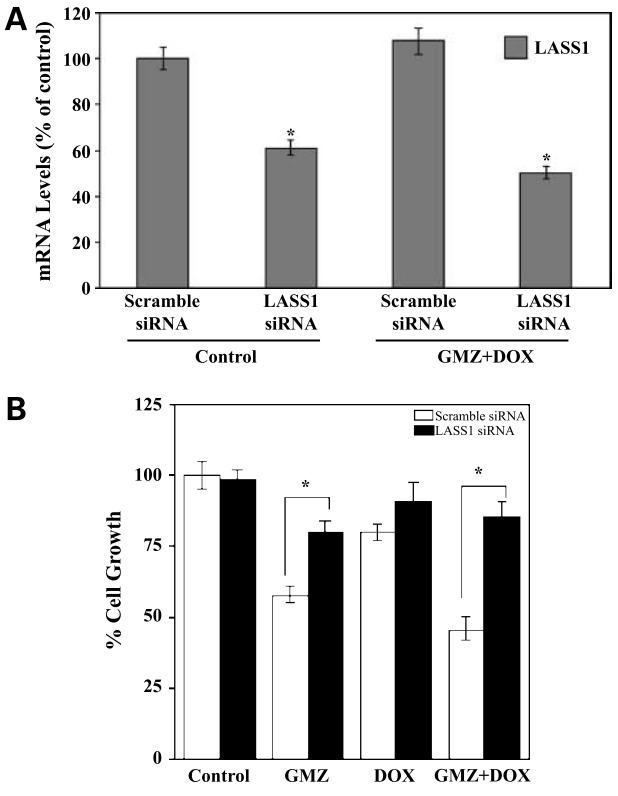

Figure 6. The role of LASS1/C_{18}-ceramide in gemcitabine/doxorubicin-induced cell death in UM-SCC-22A cells. **A**, UM-SCC-22A cells were transfected with LASS1 siRNA or scrambled (SCR, nontargeting) siRNA as described in Materials and Methods, and LASS1 mRNA expression was determined by Q-PCR. **B**, effects of LASS1 or SCR siRNAs on gemcitabine/doxorubicin-induced growth inhibition were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. The data are representative of two independent experiments done in duplicates. *Bars*, SD. Statistical analysis was done as described in Materials and Methods. *, $P < 0.05$, statistically significant.

Effects of Gemcitabine/Doxorubicin in Combination on the HNSCC Tumor Growth and/or Progression *In vivo*

To examine the efficacy of gemcitabine/doxorubicin combination *in vivo*, SCID mice bearing UM-SCC-22A xenografts were treated with these drugs alone or in combination using concentrations lower than their previously reported maximum tolerated doses, i.p. at 40 and 1 mg/kg for gemcitabine and doxorubicin, respectively, every 4 days for 32 days (8 cycles) as described in Materials and Methods. The data revealed that treatment with gemcitabine/doxorubicin in combination almost completely inhibited HNSCC tumor growth ($P < 0.05$), whereas treatment with these drugs as single agents caused a slight decrease in tumor growth, which were not statistically significant ($P > 0.05$; Fig. 8A). These data show that gemcitabine/doxorubicin combination inhibits HNSCC tumor growth and/or progression significantly both *in situ* and *in vivo*. Additionally, histopathologic analysis of tissues obtained from vital organs of these animals (such as brain, heart, kidney, lung, and liver) showed no apparent overall toxicity after treatment with gemcitabine/doxorubicin in combination (data not shown). There was also no significant weight loss in these animals after treatment with these drugs (data not shown).

Involvement of Increased Ceramide Generation in the Inhibition of HNSCC Tumor Growth in Response to Gemcitabine/Doxorubicin Treatment *In vivo*

Multiple previous studies have shown the effects of gemcitabine or doxorubicin on the generation of ceramide in various cancer cells as single agents (11, 29); however, their effects in combination on ceramide generation *in vivo* have not been examined previously. Therefore, to measure the effects of these drugs on the generation of endogenous ceramide *in vivo*, the tumors, which were confirmed to be squamous cell carcinomas by histopathologic examination after H&E staining (Fig. 8B), were removed after completion of studies, then the levels of ceramide were measured by LC/MS. Interestingly, the data showed that the levels of only C_{18}-ceramide (Fig. 8C) were elevated significantly (about 7-fold), whereas the levels of C_{16}-ceramide were decreased in the tumors of mice treated with gemcitabine/doxorubicin in combination as compared with controls (Fig. 8D), suggesting that the generation of C_{18}-ceramide might be important for drug-induced regulation of HNSCC tumor growth *in vivo*.

Because it is known that LASS1 is specifically involved in the generation of C_{18}-ceramide (17) and defects in C_{18}-ceramide generation/LASS1 pathway were reported to be involved in the pathogenesis and/or progression of tumor growth in patients with HNSCC (20), the levels of LASS1 mRNA were measured by Q-PCR in tumors extracted from animals treated with gemcitabine and doxorubicin, alone or in combination, as compared with controls. Figure 9A shows that treatment with gemcitabine/doxorubicin significantly increased (about 7-fold) the expression levels of LASS1 mRNA in HNSCC tumors grown in SCID mice, whereas there were no significant changes in mRNA levels

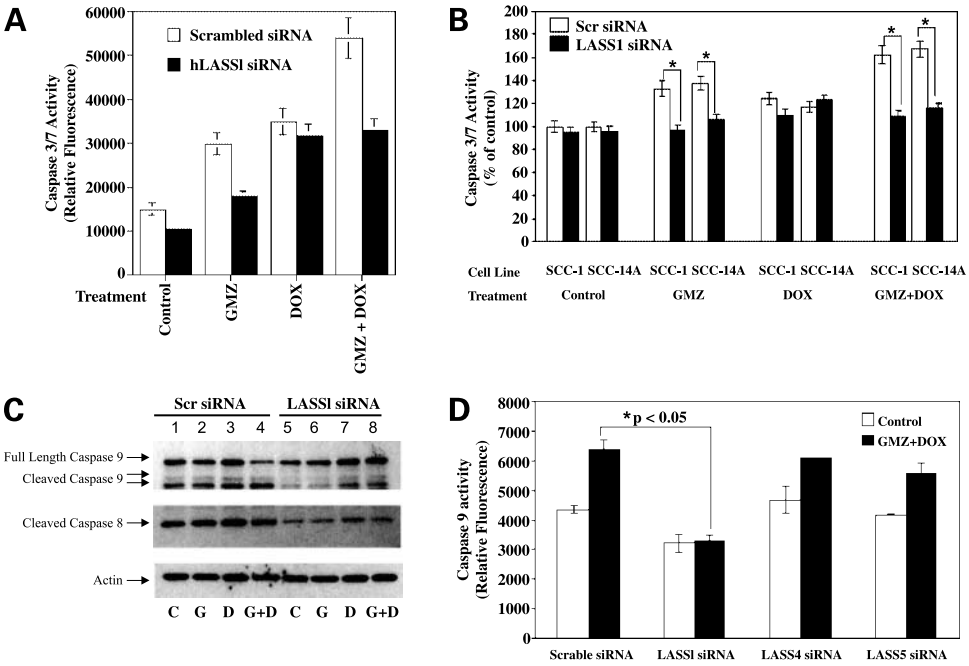

Figure 7. Role of LASS1 in gemcitabine/doxorubicin-induced caspase-3/7 and caspase-9 activation. **A,** the effects of LASS1 or SCR siRNAs on caspase-3/7 activity was determined as described in Materials and Methods in UM-SCC-22A cells. **B,** the effects of LASS1 or SCR siRNAs on caspase-3/7 activity was determined as described in Materials and Methods in UM-SCC-14A and UM-SCC-1 cells. **C,** the effects of LASS1 or LASS5 siRNAs (*lanes 5–8*) compared with SCR siRNA (*lanes 1–4*) on the activation of caspase-8 (*bottom*), or caspase-9 (*top*) in response to gemcitabine (*G*), doxorubicin (*D*), and gemcitabine/doxorubicin (*G + D*) treatments (*lanes 2–4* or *6–8*, respectively) were determined by Western blotting using antibodies against caspase-9 (full-length and cleaved forms), caspase-8 (cleaved form). β-Actin levels were used as loading controls. **D,** effects of SCR, LASS1, LASS4, or LASS5 siRNAs on gemcitabine/doxorubicin-induced caspase-9 activity were determined using caspase-9 activity assay as described in Materials and Methods. Statistical analysis was done as described in Materials and Methods. *, $P < 0.05$, statistically significant.

of LASS2, LASS3, LASS4, or LASS5 (data not shown) and LASS6 (Fig. 9A) in these tumors.

Similarly, treatment with gemcitabine/doxorubicin in combination resulted in a significant increase in the levels of LASS1 protein, whereas LASS6 protein was almost completely lost in HNSCC tumors treated with gemcitabine/doxorubicin *in vivo* (Fig. 9B, *lanes 1-5-8* and *5-8*), respectively). The data from *in vivo* experiments are in accordance with the data from *in situ* experiments, suggesting that down-regulation of LASS6 in response to gemcitabine/doxorubicin is controlled at the posttranscriptional level by an unidentified mechanism.

Thus, these data suggest that the combination of gemcitabine/doxorubicin provides an improved alternative strategy for the treatment of HNSCC *in vivo* by a mechanism possibly involving the increased generation of C_{18}-ceramide via the induction of LASS1 expression.

Discussion

Results presented in this study show that treatment with the combination of gemcitabine/doxorubicin improves the regulation of growth of HNSCC cells both *in situ* and in animal models, suggesting that this combination might offer an alternative treatment strategy against these cancers. Importantly, treatment of SCID mice bearing UM-SCC-22A xenografts with gemcitabine/doxorubicin combination significantly inhibited HNSCC tumor growth, which was concomitant with a significant increase in C_{18}-ceramide levels. In addition, further analysis of a possible role for C_{18}-ceramide, a product of LASS1 activity, in drug-induced cell death *in situ*, showed that treatment with gemcitabine/doxorubicin resulted in a significant increase in LASS1 expression and endogenous enzymatic activity of LASS1 for the generation of C_{18}-ceramide in these cells. Mechanistically, the data obtained using molecular approaches either to partially inhibit or induce LASS1 expression in these cells revealed an important role of LASS1/C_{18}-ceramide signaling in gemcitabine/doxorubicin-induced caspase-9 and caspase-3–dependent cell death. Specifically, the mechanism of action of these drugs leading to caspase-9/3–dependent mitochondrial cell death seems to involve the induction of the generation of C_{18}-ceramide by increased expression of LASS1 mRNA and enhanced (dihydro)ceramide synthase activity of LASS1 in response to gemcitabine/doxorubicin combination in HNSCC cells.

A role of LASS1 and C_{18}-ceramide in the inhibition of HNSCC tumor growth and/or cell death was defined previously (20). Specifically, it has been shown that a majority of HNSCC tumors contained lower levels of C_{18}-ceramide as compared with their normal counterparts, suggesting that defects in LASS1/C_{18}-ceramide pathway might play a role in the HNSCC tumor growth and/or

progression (20). The data presented here are in agreement with these previous results, demonstrating an important role for LASS1 via the generation of C_{18}-ceramide in drug-induced cell death both *in situ* and *in vivo* models of HNSCC. Indeed, analysis of ceramide levels in tumors obtained from patients with nonsquamous head and neck cancers in our laboratory showed lower levels of C_{16}-, C_{18}- and C_{24}-ceramides as compared with normal lung tumors (20), suggesting that the role of LASS1 and C_{18}-ceramide in the regulation of growth and therapy might be specific for squamous carcinomas of the head and neck.

The longevity assurance gene 1 (LAG1) was first discovered in yeast and found to decline as a function of increased number of cell division (14) and reviewed in ref. 33. The mammalian homologues of yeast LAG1 were later identified in human, *Caenorhabditis elegans* (19), and mouse (17), and recent independent studies have shown specific functions of mouse LASS1, LASS5, and LASS6 in the specific generation of C_{18}-, C_{16}- and C_{14}/C_{16}-ceramides, respectively (17, 30, 31). Importantly, although the yeast LAG1 requires LAC1 and Lip1 for the generation of ceramide (34), mouse LASS5 was shown to be a bona fide (dihydro)ceramide synthase without any detectable requirement for any associated proteins for its enzyme activity (31), suggesting distinct regulatory mechanisms for the ceramide synthase function of LAG1-related proteins in yeast versus mammalian cells. In addition, the role for LASS1 in the regulation of growth and apoptosis of neuronal ceroid lipofuscinosis, a hereditary childhood neurologic disorder, has been recently shown, confirming the distinct and important roles of LAG1-generated ceramide in various biological disorders (23).

The involvement of ceramide metabolism in the regulation of cell growth and in chemotherapy-induced cell death suggests the use of novel therapeutic approaches to mimic ceramide cellular actions or to elevate endogenous ceramide levels. Conventional short-chain ceramides, such as C_2- and C_6-ceramides, were shown to induce cell death in a variety of cancers. However, because of poor solubility and bioavailability of the conventional ceramides, novel pyridinium ceramides (Pyr-Cer) have been synthesized with greater water solubility and cell permeability (26, 35, 36). In addition, the positively charged pyridinium ring of these novel ceramides permits them to target and accumulate in negatively charged intracellular compartments, especially the mitochondria and nucleus (37). L-t-C_6-Pyr-Cer, alone or in combination with gemcitabine, has been shown *in vivo* to preferentially accumulate in the tumor site and modulate telomerase, decrease telomeric length, and inhibit HNSCC tumor growth (26).

Chemotherapeutic agents, such as daunorubicin, vincristine, or gemcitabine, usually elevate endogenous ceramide levels via the *de novo* pathway, or activation of SMases in many human cancer cells (11, 29). In addition, Sphingomyelin/gemcitabine combination synergistically inhibits pancreatic tumor growth *in vivo* (38). The cytotoxic effect of doxorubicin was also enhanced in combination with SM

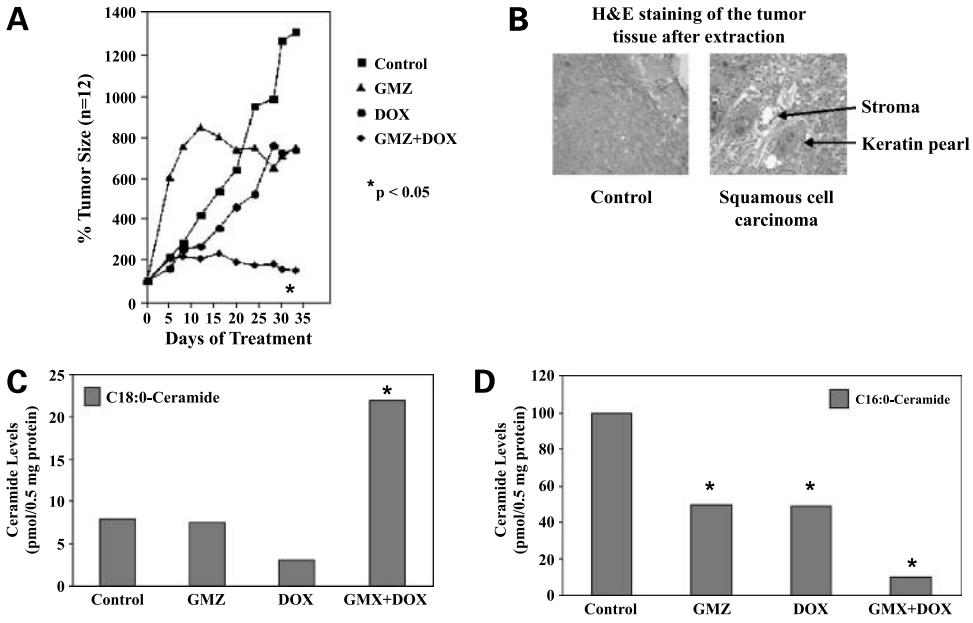

Figure 8. Improved growth-inhibitory effects of gemcitabine, in combination with doxorubicin, on UM-SCC-22A cells *in vivo* xenografts on SCID mice. **A,** UM-SCC-22A xenografts were generated on the flanks of SCID mice, and the mice were injected i.p. with gemcitabine, doxorubicin, or gemcitabine and doxorubicin in combination as described in Materials and Methods at every 4 d for 32 d. Tumor sizes were measured every 4 d, and the tumor volumes were calculated based on the formula: $V = \text{Length} \times \text{Width}^2/2$. Statistical analysis was done as described in Materials and Methods. *, $P < 0.05$, statistically significant. **B,** H&E staining of the tumor tissues after extraction confirmed squamous cell carcinomas. **C** and **D,** the levels of $C_{18:0}$- and $C_{16:0}$-ceramides, respectively, of the extracted tumor tissues were determined by LC/MS and normalized to 0.5 mg of total proteins. Statistical analysis was done as described in Materials and Methods. *, $P < 0.05$, statistically significant.

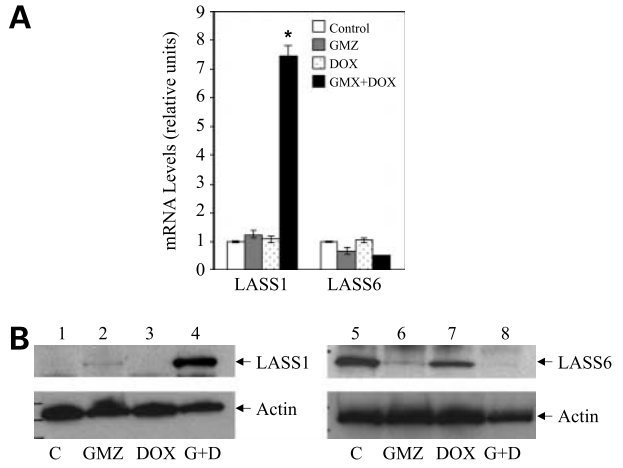

Figure 9. Modulation of mRNA expression levels of LASS genes and LASS1 and LASS6 protein levels in response to gemcitabine/doxorubicin-induced inhibition of tumor growth in UM-SCC-22A xenografts *in vivo*. **A,** mRNA levels of LASS1 and LASS6 were determined by Q-PCR as described in Materials and Methods. At least three experiments were done in triplicates; *bars,* SD. Statistical analysis was done as described in Materials and Methods. *, $P < 0.05$, statistically significant. **B,** levels of LASS1 and LASS6 proteins of the extracted tumor tissues were determined by Western blotting. Actin levels are used as loading controls.

due to the increased cellular uptake of doxorubicin through the plasma membrane (39).

In this study, we show that gemcitabine/doxorubicin combination treatment supra-additively inhibited HNSCC growth both *in vivo* and *in situ*. On the other hand, doxorubicin, when used at high concentrations in combination with gemcitabine, had an antagonistic effect, determined in isobologram studies. The reasons for the antagonistic effects of doxorubicin at high concentrations are still unknown and should be further studied before gemcitabine/doxorubicin combination is used in the clinic.

Nevertheless, the role for these LASS-member proteins in the regulation of drug-induced cell death has not been shown previously, and the results presented here show, for the first time, that LASS1/C_{18}-ceramide, plays an important role in drug-induced cell death and caspase activation in HNSCC cells. The precise role of LASS1/C_{18}-ceramide in mitochondrial integrity and/or function leading to cell death in the absence or presence of chemotherapeutic agents, however, is still unknown and needs to be determined. One of the possible downstream mechanisms that might play a role in LASS1/C_{18}-ceramide–mediated caspase activation and cell death in response to chemotherapy may be the alternative splicing of caspase-9 mRNA to generate its proapoptotic form (29, 40). This, however, needs to be further evaluated. The data presented here showed that LASS1 plays an important role in chemotherapy-induced activation of caspase-9 and caspase-3/7. Interestingly, inhibition of LASS1 expression by siRNA seemed to inhibit cleavage/activation of caspase 8 as compared with scrambled control (Fig. 7C, *lanes 1* and *5*), respectively, which was not affected by gemcitabine/doxorubicin. It should also be noted that using compounds that do not affect C_{18}-ceramide generation via LASS1 in combination might still exert synergistic effects by mediating distinct cellular targets and mechanisms that are not controlled by C_{18}-ceramide signaling.

Interestingly, in addition to increased levels of C_{18}-ceramide, significantly reduced levels of C_{16}-ceramide were observed in SCID mice bearing UM-SCC-22A xenografts after treatment with gemcitabine/doxorubicin. These data are consistent with our previous observation, which showed that whereas the majority of tumors obtained from patients with HNSCC contained lower levels of C_{18}-ceramide, higher levels of C_{16}-ceramides were detected in these tumor tissues when compared with their normal tissues (20). Thus, taken together, these data implicate that increased levels of C_{18}- and C_{16}-ceramides may play opposing roles in the regulation of tumor growth and/or cell death in HNSCC cells (20, 41). Therefore, the identification of specific roles and downstream targets of these ceramides with different fatty acid chain lengths (C_{16}-, C_{18}-, and C_{24}-ceramides), which are known to be products of distinct LASS-member proteins, in various different types of cancer cells is extremely important and of great interest in our laboratories. In fact, distinct roles of different ceramides have been shown previously. For example, the role for C_{16}-ceramide in apoptosis upon induction by immunoglobulin M via *de novo* pathway (5) and C_{24}-ceramide in cell cycle arrest via the overexpression of neutral sphingomylinase-2 (N-SMase-2) in MCF-7 cells (42), were reported previously.

In summary, these results show that development of new chemotherapeutic treatments that target specific ceramide pathways might help develop novel strategies for the treatment of HNSCC in the clinic.

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