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Advances in Brief

Human Mesothelioma Samples Overexpress Both Cyclooxygenase-2 (COX-2) and Inducible Nitric Oxide Synthase (NOS2): *In Vitro* Antiproliferative Effects of a COX-2 Inhibitor

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Abstract

Accumulating data demonstrate overexpression of both inducible NO synthase (NOS2) and cyclooxygenase-2 (COX2) in many epithelial neoplasias. In addition, cyclooxygenase inhibitors have been shown to have antineoplastic and prophylactic efficacy against human colon cancer and in mouse models of this disease. Mesothelioma arises in a context of asbestos exposure and chronic inflammation, which would be expected to enhance the expression of these inducible enzymes. This study demonstrates that both inducible enzymes were expressed in 30 human mesothelioma tissues but were not detectable in nonreactive mesothelial tissues from the same individuals. In contrast, areas of reactive mesothelial cells stained positively for these enzymes. *In vitro* exposure of human mesothelioma cell lines to the COX2 inhibitor, NS398, revealed dose- and time-dependent antiproliferative activity, whereas the NOS2 inhibitor, 1400W, had no detectable inhibitory effect. Surprisingly, nonmalignant human mesothelial isolates expressed both NOS2 and COX2 *in vitro* at the same level as mesothelioma cell lines but were less sensitive to NS398 inhibition. This finding indicates that these nonmalignant isolates may retain properties of reactive mesothelial cells and suggests that targets in addition to COX2 may be involved in the antiproliferative response of mesothelioma cell lines. These results have clinical significance because of the selective activity of the drug coupled with the therapeutic resistance and poor prognosis of mesothelioma. The findings presented here suggest that further preclinical studies of these inhibitors in animal models of mesothelioma would be of great interest.

Introduction

The development of MM[2] is associated, in most cases, with a history of asbestos exposure (1). Accumulating reports of the presence of DNA encoding SV40 T antigen or of SV40 T antigen protein expression (reviewed in Ref. 2) suggest that the presence of this gene may also be associated with mesothelioma. Although molecular mechanisms of asbestos tumorigenicity have not been elucidated, research has shown that asbestos exposure generates reactive oxygen species and reactive nitrogen species and activates macrophages and other cell types to produce these compounds as well as cytokines and growth factors (reviewed in Ref. 3). Furthermore, the deposition of insoluble amphibole fibers results in a chronic inflammatory state in exposed individuals (4).

The existence of inflammation has been associated with up-regulation of the inducible species of both NOS2 and COX2 (5) and is associated with an increased risk of cancer (6). Both *in vitro* and *in vivo* experiments have demonstrated that asbestos induces expression of active NOS2 in rat alveolar macrophages and pleural mesothelial cells (7, 8). It has been shown that COX2 is induced by inflammatory cytokines and that cultured human mesothelial cells contain cyclooxygenase activity (9). Indeed, *in vitro* experiments demonstrated that cyclooxygenase expression in mesothelial cells is increased by exposure to tumor necrosis factor-α, interleukin 1β, or macrophage-conditioned medium (10). Recent data specifically demonstrate that COX2 is induced in rat mesothelial cells during rat carrageenin-induced pleurisy (11).

Mesothelioma has proved resistant to classical chemotherapeutic and radiation regimens (12). This property may be modeled by the resistance demonstrated by mesothelioma cell lines to multiple inducers of apoptosis (13) and to the increased resistance to asbestos-induced apoptosis shown by mesothelial cell strains surviving asbestos exposure (14). COX2 overexpression has been linked to resistance to apoptotic signals and COX2-specific NSAIDs have been shown to increase apoptosis (15–17). Furthermore, NSAIDs have been shown to be useful as prophylactic agents for human colon cancer (18). It was, therefore, of interest to examine whether NOS2 and/or COX2 might be expressed differentially in mesothelioma as compared with mesothelial cells and whether inhibitors of these enzymes would be antiproliferative for mesothelioma cell lines.

We report here that, in a study of 30 human mesothelioma samples and uninvolved lung controls, both NOS2 and COX2 are overexpressed in mesothelioma. Furthermore, the COX2-specific NSAID, NS398 but not the NOS2-specific, 1400W, demonstrated antiproliferative activity *in vitro* for human mesothelioma cell lines.

Materials and Methods

Materials

Tissue Samples. Samples from 30 patients (mean age, 58 years; range, 30–78 years) with histological and clinical diagnoses of MM were examined in this study. These samples were flash frozen in liquid nitrogen at surgery. All patients reported histories of asbestos exposure. Clinical staging at surgery revealed stage I (3), stage II (7), and stage III (20) disease according to the criteria reported by Rusch *et al.* (19). Surgical treatments included pleurectomy, tumor debulking, or extrapleural pneumonectomy. Uninvolved deep-lung specimens were obtained at surgery. Survival history (29 of 30) includes 5 survivors (6–72 months postoperative; mean age, 32.6 years), 19 MM-related deaths, 3 deaths from operative or postoperative complications, and 2 deaths from aspiration pneumonia within 2 months of surgery.

Cell Lines. The MM cell lines used in this study have been characterized previously and were cultured as described (20, 21). These lines were uniformly negative for SV40 T antigen by immunohistochemistry. Individual human mesothelial cell strains were obtained from noncancerous donors through a protocol with the CHTN (Philadelphia, PA). Cells were cultured as reported previously (14).

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[2] The abbreviations used are: MM, malignant mesothelioma; NOS2, inducible nitric oxide synthase; COX2, cyclooxygenase-2; NSAID, nonsteroidal anti-inflammatory drug; CHTN, Cooperative Human Tissue Network; FACS, fluorescence-activated cell sorter.

Immunohistochemistry

Tissue sections (4 μm) from samples embedded in paraffin blocks were cut and mounted on electrically charged glass slides. Sections were heated at 60° for 45 min, deparaffinized in three changes of xylene, and fixed in a decreasing ethanol series (100 to 50%) for 5 min/solution. Samples were treated for 30 min with 3% H_2O_2 to block endogenous peroxidase activity. Antigen retrieval involved treatment with an antigen retrieval solution (BioGenex, San Ramon, CA) in a microwave oven (140 joules) for 30 min. Tissue sections or 10% formalin-fixed cell lines were incubated overnight at 4°C in moist chambers with monoclonal antibodies to NOS2 (N32020) or COX2 (C22420; Transduction Laboratories, Lexington, KY) at dilutions of 1:125 and 1:25, respectively. Binding of the primary antibody was detected by incubation for 45 min at room temperature with biotinylated universal secondary antibody (1:200; VectaStain ABC-AP kit; Vector Laboratories, Burlingame, CA), followed by incubation with streptavidin peroxidase as directed by the manufacturer. Chromogenic development was obtained by immersion in 3,3′- diaminobenzidine solution (0.25 mg/ml in 3% hydrogen peroxide). Slides were counterstained with Harris' hematoxylin, and coverslips were applied after the application of mounting medium. Results are reported as follows: 0, <10% reactivity; +1, 10–25%; +2, 25–50%; +3, 50–90%; and +4, >90% according to criteria established by the College of American Pathologists (22).

NOS2 and COX2 Inhibitor Studies

Cell lines were plated at 5×10^4 cells/dish in 60-mm dishes 1 day before application of test medium. At day 1, medium containing various concentrations of inhibitor or an equivalent solvent blank was applied. Medium was changed every 2 days because of inhibitor instability. Inhibitors tested were 1400W, a specific inhibitor of NOS2 (Ref. 23; Biomol Research Laboratories, Plymouth Meeting, PA) shown to inhibit tumor growth *in vivo* (24) and NS398, specific for COX2 (Ref. 25; Alexis Biochemicals, San Diego, CA). At the appropriate time point, cells were removed from dishes by trypsinization and counted in a hemacytometer. When cells were to be analyzed by FACS, cells from supernatant medium before trypsinization were recovered by centrifugation and pooled with adherent cells before alcohol fixation and treatment with propidium iodide. FACS analysis was performed on a Becton Dickinson FACSCalibur instrument with data acquisition by the Cell Quest program. Analysis of cell cycle distribution was performed using the Modfit program.

Results

Histology and Immunohistochemistry

Tumor Specimens. Table 1 presents summary results from immunohistochemical analysis of 30 MM specimens and deep-lung controls. Histologically, based on H&E (2) staining, tumor specimens contained 23 epithelial, 3 sarcomatoid, and 4 biphasic patterns. Control tissue displayed normal histology with no reactive or neoplastic changes involving the pleural lining in 26 of 30 samples. Among this group, summarized in Table 1, adjacent pulmonary tissues showed acute hemorrhage in 3 specimens (nos. 2, 16, and 22), acute pneumonia in 1 specimen (no. 13), and foreign body reaction in 1 specimen (no. 26). In the remaining 4 specimens, 3 displayed reactive mesothelial cells (nos. 4, 14, and 20) and 1 showed atypical mesothelial hyperplasia with a likely focus of MM (no. 29). Additionally, 1 control specimen with pulmonary hemorrhage displayed organizing pneumonia (no. 22). All specimens were stained with monoclonal antibodies for the inducible enzymes NOS2 and COX2. All tumor specimens were strongly positive for COX2 whereas 26 of 30 were positive for NOS2 (Table 1; Fig. 1). In addition to staining in mesothelioma cells, mononuclear inflammatory cells were also positive for both NOS2 and COX2. In contrast, both COX2 and NOS2

Table 1 *Clinicopathologic and immunohistochemical characterization of MM tumors and control lung samples*

Case	Sex	Age	Stage (tumor)	Histology (tumor)	NOS2 (tumor)	COX2 (tumor)	Histology (C-1)[h]	NOS2 (C-1)	COX2 (C-1)
1	M	67	III	Biphasic	+4[a]	+2	Normal		
2	M	53	III	Epithelial	+4	+3	H[b]		
3	F	63	I	Sarcomatoid	+4	+2	Normal		
4	M	68	II	Epithelial	+4	+3	RMH[c]	+3	+2
5	M	49	III	Epithelial		+3	Normal		
6	M	78	I	Epithelial	+4	+2	Normal		
7	F	48	II	Epithelial	+3	+4	Normal		
8	F	45	I	Epithelial	+4	+3	Normal		
9	M	45	III	Epithelial	+4	+2	Normal		
10	M	69	III	Biphasic	+4	+2	Normal		
11	M	30	II	Epithelial		+2	Normal		
12	F	58	III	Epithelial	+4	+4	Normal		
13	M	50	III	Epithelial	+4	+4	Acute Pn[d]		
14	M	52	III	Epithelial	+4	+4	RMH[c]	+2	+3
15	M	59	III	Epithelial	+4	+3	Normal		
16	M	63	III	Epithelial	+2	+3	H[b]		
17	F	52	III	Epithelial	+4	+2	Normal		
18	M	63	II	Epithelial	+4	+4	Normal		
19	M	62	III	Epithelial		+2	Normal		
20	M	72	III	Epithelial	+2	+4	RMH	+2	
21	M	68	III	Epithelial	+3	+4	Normal		
22	F	42	I	Epithelial		+4	Hb/OPn[e]		
23	M	65	III	Epithelial	+4	+4	Normal		
24	M	60	III	Epithelial	+4	+4	Normal		
25	M	65	III	Biphasic	+4	+4	Normal		
26	M	62	II	Epithelial	+4	+4	FB Rxn[f]		
27	F	46	III	Sarcomatoid	+4	+4	Normal		
28	M	61	III	Epithelial	+4	+2	Normal		
29	M	76	III	Biphasic	+4	+2	ATMH/MM[g]	+2	+4
30	M	59	III	Sarcomatoid	+4	+2	Normal		

[a] Number scores were assigned according to criteria established by the College of American Pathologists as described in "Materials and Methods."
[b] H, hemorrhage.
[c] RMH, reactive mesothelial hyperplasia.
[d] Pn, pneumonia.
[e] OPn, organizing pneumonia.
[f] FB Rxn, foreign body reaction.
[g] ATMH/MM, atypical mesothelial hyperplasia/malignant mesothelioma.
[h] C-1, control lung sample.

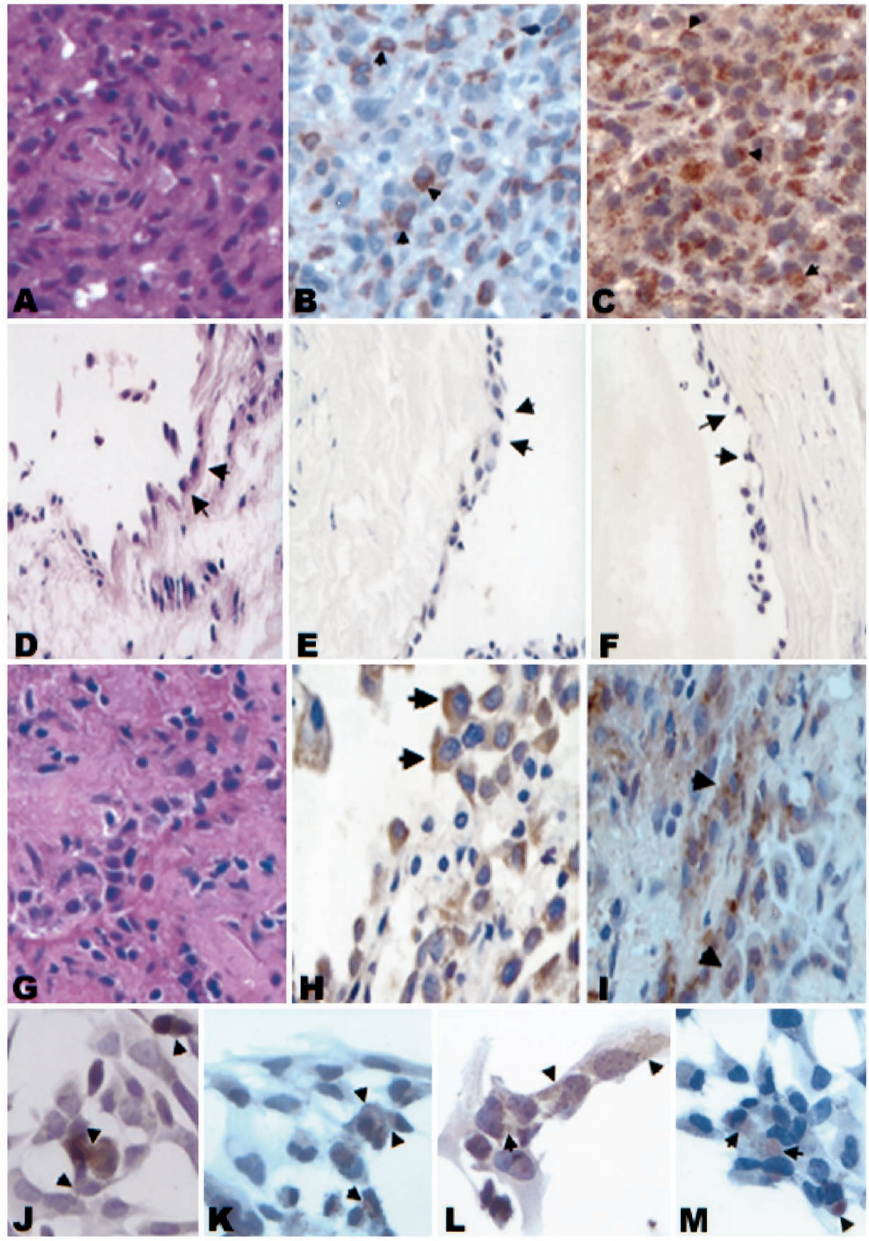

Fig. 1. COX2 and NOS2 expression in MM, control lung samples, reactive mesothelial hyperplasia, and in human mesothelial cell strains and human mesothelioma cell lines. A paraffin-embedded biphasic mesothelioma specimen as seen on H&E (*A*) was stained as described in "Materials and Methods" with anti-COX2 (*B*) and anti-NOS2 (*C*). *Arrowheads* indicate cytoplasmic reactivity with the respective antibodies. ×250. A sample of tissues from a nonneoplastic control lung pleural surface from the patient (*A–C*) is shown. *D*, H&E staining demonstrates a normal mesothelial lining. Staining with anti-COX2 (*E*) and anti-NOS2 (*F*) was negative. ×275. An example of immunohistochemical staining of areas of reactive mesothelial hyperplasia found in control specimens is shown. *G*, H&E revealed an area of hyperplasia with reactive mesothelial cells, which revealed immunoreactivity to anti-COX2 (*H*) and anti-NOS2 (*I*). ×250. A human mesothelial cell strain (*J* and *L*; 10141) and a mesothelioma cell line (*K* and *M*; M33K) show positive staining with antibodies to COX2 (*J* and *K*) and NOS2 (*L* and *M*). As in tumor specimens, staining was cytoplasmic and granular. *J*, ×195; *K*, ×250; *L*, ×265; *M*, ×230.

were undetectable in normal mesothelial cells in the control tissue (26 of 30). Specimens (nos. 4, 14, 20, and 29), which contained reactive mesothelial cells or atypical mesothelial hyperplasia showed mesothelial cells positive for NOS2 in all cases and for COX2 in 3 cases (nos. 4, 14, and 29).

Fig. 1 presents representative examples of material from Table 1. Panels show H&E, COX2, and NOS2 staining, respectively, in MM (*A–C*) and a control sample (*D–F*) from an individual with a biphasic MM containing both epithelial and spindeloid cells. The pattern of reactivity displayed by both COX2 and NOS2 was granular and cytoplasmic (*arrowheads*; *B* and *C*). Mesothelial cells in control tissue (*D*) are negative for both COX2 (*E*) and NOS2 (*F*) staining (*arrowheads*). Mesothelial cells in an area of reactive mesothelial hyperplasia (*G*) stained positively for COX2 (*H*) and NOS2 (*I*). Nonhyperplastic regions were negative for staining by either antibody.

Cultured Cells. To evaluate the expression of COX2 and NOS2 in human mesothelioma cell lines and human mesothelial cell strains in culture, cells grown on slides were fixed and stained with the monoclonal antibodies used for tumor samples. Eight human mesothelioma cell lines (Vamt-1, M9K, M10K, M14K, M25K, M28K, and M33K) and four cultures derived from nonmalignant pleural fluids (CHTN 11027,11032, 11039, and 10141) were tested. Representative areas of slides from a mesothelial cell strain (Fig. 1, *J* and *L*) and a MM cell line (Fig. 1, *K* and *M*) are shown in Fig. 1. Although only MM cell lines were expected to be positive, all 12 cultures showed some expression of both COX2 and NOS2. Cell strain 10141 was tested as a third passage after 6 months of cryopreservation but was not distinguishable from the other three strains with respect to staining for NOS2 and COX2. The levels of COX2 detected by immunohistochemistry in both mesothelioma cell lines and human individual cell strains were not detectable by Western blotting, which detected 50 ng of an ovine COX2 standard. Interestingly, the pathology reports received on all four primary pleural fluid specimens indicated that "reactive mesothelial cells" were present in the fluids. Thus, the nonmalignant

Table 2 *NS398 inhibition of mesothelial cells and mesothelioma cell lines*[a]

Cell	Cell type	Solvent blank	100 μM NS398	% inhibition
CHTN 18650	Primary	8.7×10^4 (±1.0)	7.55 ± 10^4 (±1.3)	13
VAMT-1	MM	2.53×10^6 (±0.36)	7.53×10^5 (±0.45)	70
M10K	MM	6.26×10^4 (±3.0)	2.95×10^4 (±2.9)	53
M14K	MM	1.67 ± 10^6 (±2.0)	6.91×10^5 (±2.8)	59
M33K	MM	1.33 ± 10^6 (±0.13)	5.0×10^5 (±0.52)	63

[a] Cells were seeded in triplicate at 5×10^4 cells/60-mm dish and treated with 100 μM NS398 as described in "Materials and Methods." At 6 days, all dishes were trypsinized, and viable cells were counted. Data show the average cell number ± SD.

mesothelial cells, which proliferated in culture, may represent a hyperplastic state cited as "reactive" by the pathologist.

Effects of COX2 and NOS2 Inhibitors. Effects of both COX2 and NOS2 inhibitors on human mesothelioma cell lines and mesothelial cell strains were evaluated. The mesothelioma cell lines tested expressed wild-type p53 with the exception of the p53 null VAMT-1 cell line (20).

Table 2 shows the results obtained with 100 μM NS398, a COX2 specific inhibitor (25). Interestingly, the MM cell lines are more severely inhibited than the mesothelial cell strain. However, immunohistochemistry did not reveal differences in COX2 expression among these cells (Fig. 1). Because the mesothelial cell strain was expected to display a slower growth rate than MM cell lines, M10K, a slow-growing MM line, was included in this analysis. The MM cell line was 4-fold more severely inhibited than the mesothelial cell strain. In a parallel experiment using 50 μM 1400W, a NOS2-specific inhibitor (23) on all cell lines, no inhibition was detected at 6 days of treatment (data not shown).

The dose responsiveness and time dependence of NS398 inhibition were next examined using the VAMT-1 human MM cell line. The same cell line was tested in parallel with concentrations of 1400W from 0.1 to 100 μM. The percentage of control growth shown by samples treated with 1400W ranged from 81 to 103%, and treatment did not produce a significant effect. At 100 μM, the cell number was 90% of the solvent control. Results for NS398 are shown in Fig. 2. The inhibition shows a clear dose dependence with an ID_{50} of 87 μM (Fig. 2*A*). This dose was used in the time course shown in Fig. 2*B* in which the effect of NS398 on VAMT-1 cells was compared with its effect on an individual human mesothelial isolate (CHTN 18833) different from that shown in Table 2. Consistent with earlier results, the MM cell line was inhibited to a greater extent than the individual human mesothelial strain.

Because it has been reported that treatment with COX2 inhibitors can induce apoptosis (26), we used FACS analysis of propidium iodide-stained cells to examine VAMT-1 cells treated for 6 days at 85 μM NS398 for the presence of an apoptotic cell peak and for cell cycle distribution parameters. Table 3 shows the results of this analysis in comparison with cells treated with a solvent control. No sub-G_1 fraction of apoptotic cells was detected in either sample (data not shown). Treated cells showed a clear reduction in the S-phase fraction and a large increase in cells in the G_2-M phase of the cell cycle. These results are consistent with the antiproliferative effect of NS398 demonstrated earlier and suggest that this effect is not mediated by increased apoptosis.

Discussion

The data presented here demonstrate that cells in mesothelioma tissue, as opposed to normal mesothelial linings, express detectable levels of the inducible enzymes, NOS2 and COX2. Both spindeloid and epithelial forms of mesothelioma as well as regions of reactive mesothelial hyperplasia expressed these enzymes (Table 1; Fig. 1). It has been shown that NOS2 is induced in rat mesothelial cells by exposure to asbestos in the presence of interleukin 1β (8). In addition, asbestos exposure has been shown to lead to activation of nuclear factor-κB and activator protein-1 transcription factors (27), which have been implicated in COX2 expression (28, 29). It is interesting that the areas containing normal mesothelial cells do not show expression of the inducible enzymes. Perhaps exposure to inducers is sensitive to concentration gradients and remains a localized phenomenon. In this regard, examination of *in vitro* propagated individual human mesothelial isolates and human mesothelioma cell lines also showed expression of these enzymes (Fig. 1). This finding suggests that the individual isolates represent a cell similar to that seen in the reactive mesothelial hyperplasia *in vivo* (Fig. 1), or alternatively, that the optimized growth conditions used for *in vitro* propagation (30) are sufficient to induce expression of NOS2 and COX2 in human mesothelial cells.

Because mesothelioma remains a tumor type that is resistant to

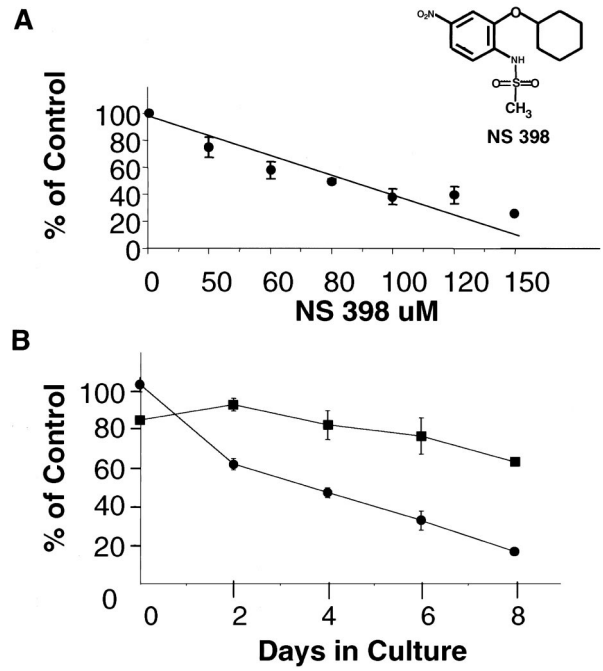

Fig. 2. Antiproliferative effects of NS398. The chemical structure of NS398 is shown above the dose and time dependence data. *A*, dose dependence of NS398 inhibition. VAMT-1 cells were exposed to varying doses of NS398 or solvent control for 6 days. Media were changed on days 2 and 4. On day 6, cells were trypsinized and counted, and data represent the relation of NS398 treated to solvent control cell number. *B*, time dependence of NS398 inhibition. VAMT-1 (●) or a human mesothelial cell strain (■) were exposed to 87 μM NS398 or solvent control for the indicated times with media changes every 2 days. Cells were trypsinized and counted as in *A*.

Table 3 *Effect of NS398 on cell cycle distribution*[a]

		G_0-G_1	*S*	G_2-M
Solvent blank	Average (n = 6)	57.56	23.40	19.04
	SD	1.25	2.08	2.34
NS398	Average (n = 6)	55.38	17.58	27.05
	SD	1.09	0.90	0.95

[a] VAMT-1 cells were exposed to 87 μM NS398 or a solvent control for 6 days and harvested for analysis of apoptotic fraction and cell cycle distribution parameters as described in "Materials and Methods." Collected cells were fixed in 70% EtOH and treated with propidium iodide. Analyses were performed on a Becton Dickinson FACS-Calibur instrument with data collection by the Cell Quest program. Analysis of cell cycle distribution parameters was performed using the ModFit program.

therapy (12), it was of interest to test both COX2- and NOS2-specific inhibitors for antiproliferative effects on human mesothelioma cell lines. Cell lines tested expressed wild-type p53 with the exception of the p53 null VAMT-1 and were negative for expression of SV40T (20). The NOS2 inhibitor, 1400W, was without effect on cellular proliferation. In contrast, NS398, a COX2-specific inhibitor, proved to be antiproliferative for mesothelioma cell lines. Interestingly, the p53 null cell line, VAMT-1, was most inhibited (Table 2), suggesting that p53-mediated antiproliferative effects are not critical for NS398 inhibition. Although individual human mesothelial isolates were also inhibited, the effect was much weaker (Table 2; Fig. 2). This result was surprising in view of the finding that mesothelial isolates could not be distinguished from mesothelioma cell lines by immunostaining for COX2 (Fig. 1). In addition, FACS analysis of cell cycle parameters revealed that mesothelioma cells treated with NS398 did not show apoptosis but revealed a decreased S-phase fraction and an increase in the G_2-M fraction (Table 3). This result contrasts with a report that NS398 increases apoptosis in colon cancer cells independently of COX expression but does not alter cell cycle parameters (31). It is likely that the mechanisms and cellular targets involved in NSAID action will differ in varying cancer progenitor cell types.

These data suggest that inhibition of COX2 does not account for all of the antiproliferative activity that NS398 demonstrates for mesothelioma cell lines and are in agreement with reports indicating that the antineoplastic effects of NSAIDs are not solely attributable to inhibition of COX2. Colon cancer cells lacking COX activity are effectively growth inhibited by NSAIDs (31, 32), as are COX1 and COX2 null mouse embryo fibroblasts (33). Recent data identify peroxisome proliferator-activated receptor δ transcriptional activation as a COX-independent target for repression by NSAIDs in colon cancer cells (34). Additional targets may well be involved in cancers arising from other cell types in which different pathways may be critical for growth control.

In summary, the data reported here indicate that NOS2 and COX2 overexpression is common in human mesothelioma. Furthermore, COX2 inhibitors demonstrate selective antiproliferative activity *in vitro*. In view of the median survival of 8–18 months (12) from a diagnosis of MM, animal trials of NSAIDs would be of great interest. Even inhibitors of NOS2 that did not show *in vitro* antiproliferative activity might be active *in vivo* because of paracrine effects on angiogenesis or generation of cytokines. If NSAID treatment were efficacious, it might be considered as a prophylaxis for individuals known to have high exposure to asbestos. These therapeutic possibilities would be of great interest for application to this lethal cancer with an identifiable at-risk population.

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