

CD30 Is a Potential Therapeutic Target in Malignant Mesothelioma

Snehal Dabir¹, Adam Kresak², Michael Yang², Pingfu Fu³, Gary Wildey¹, and Afshin Dowlati¹

Abstract

CD30 is a cytokine receptor belonging to the TNF superfamily (TNFRSF8) that acts as a regulator of apoptosis. The presence of CD30 antigen is important in the diagnosis of Hodgkin disease and anaplastic large cell lymphoma. There have been sporadic reports of CD30 expression in nonlymphoid tumors, including malignant mesothelioma. Given the remarkable success of brentuximab vedotin, an antibody–drug conjugate directed against CD30 antigen, in lymphoid malignancies, we undertook a study to examine the incidence of CD30 in mesothelioma and to investigate the ability to target CD30 antigen in mesothelioma. Mesothelioma tumor specimens ($N = 83$) were examined for CD30 expression by IHC. Positive CD30 expression was noted in 13 mesothelioma speci-

mens, primarily those of epithelial histology. There was no significant correlation of CD30 positivity with tumor grade, stage, or survival. Examination of four mesothelioma cell lines (H28, H2052, H2452, and 211H) for CD30 expression by both FACS analysis and confocal microscopy showed that CD30 antigen localized to the cell membrane. Brentuximab vedotin treatment of cultured mesothelioma cells produced a dose-dependent decrease in cell growth and viability at clinically relevant concentrations. Our studies validate the presence of CD30 antigen in a subgroup of epithelial-type mesothelioma tumors and indicate that selected mesothelioma patients may derive benefit from brentuximab vedotin treatment. *Mol Cancer Ther*; 14(3); 740–6. ©2015 AACR.

Introduction

Malignant mesothelioma is a locally aggressive tumor with the most common form being malignant pleural mesothelioma, representing approximately 70% of all mesothelioma diagnoses. Diagnosis at an early stage of the disease is often difficult because of its nonspecific signs and symptoms, and consequently a large majority of the patients are initially diagnosed at an advanced stage. Mesothelioma is associated with occupational asbestos exposure in most industrialized countries (1). Many emerging countries have yet to prohibit asbestos usage, which predicts that a great number of the patients will suffer from this disease in the future. In addition, there is also growing concern that the widespread use of nano-sized particles for medical and industrial purposes may create new risks for developing mesothelioma. Treatment for advanced malignant mesothelioma is composed of combination chemotherapy containing platinum and pemetrexed (2).

Since approval of this regimen for mesothelioma, there have been no therapeutic advances for the treatment of this disease. Thus, there is an urgent need to develop new therapeutic strategies against this disease.

Antibody-based therapies and immunotoxins have shown remarkable therapeutic activities in various tumors and might represent a promising approach against mesothelioma (3). This approach targets antigens specifically expressed on cancerous cells, resulting in an increased therapeutic efficacy with minimal systemic toxicity. CD30 is a member of the TNF receptor super family (TNFRSF8) and is highly expressed in lymphoma with limited expression in healthy tissues, making it an ideal target for antibody-based therapies (4–8). Recently, in clinical trials for relapsed/refractory Hodgkin lymphoma and anaplastic large cell lymphoma (9, 10), brentuximab vedotin demonstrated high response rates as a single agent leading to its accelerated approval by the FDA for treatment of these lymphomas under the brand name Adcetris. Brentuximab vedotin is an antibody–drug conjugate, which couples an anti-CD30 antibody (SGN-30) to the synthetic anti-tubulin agent MMAE (11).

Although several reports have identified CD30 antigen in other types of solid tumors, including malignant mesothelioma (4, 5, 12), these findings have not been systematically followed up, which is important for broadening the family of CD30-positive cancers that are potential targets for brentuximab vedotin. Here, we validate the presence of CD30 in a panel of mesothelioma tumors by IHC and demonstrate its cell surface location in mesothelioma cell lines by confocal microscopy and flow cytometry. Importantly, the efficacy of brentuximab vedotin on mesothelioma cell lines is demonstrated using clinically relevant drug concentrations.

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Materials and Methods

Cell lines

The human small cell lung cancer cell line H526 and mesothelioma cell lines H2052, H2452, 211H, and H28 were purchased from the ATCC within the last 3 years and used at a passage of ≤ 7 . These thoracic cell lines were not authenticated because of their direct purchase from the ATCC and low passage number. Karpas cells were a gift from Dr. H. Koon (Case Comprehensive Cancer Center, Cleveland, OH) and while not authenticated, served as a positive control and responded to drug treatment in accordance with published reports (9, 10, 13, 14). Cell lines were maintained in DMEM/Ham's F-12 medium supplemented with 10% (v/v) FBS (Hyclone; ThermoFisher Scientific) in a 5% CO₂ humidified incubator at 37°C.

Confocal microscopy

Mesothelioma cells were seeded onto a glass-bottomed culture dish (MatTek Corp.). Cells were fixed with 4% formaldehyde diluted in PBS. After 1 hour blocking with 1% BSA in PBS, cells were incubated with a rabbit polyclonal anti-CD30 antibody (Novus Biologicals, NBP1-72175; 1:100) at room temperature for 2 hours and washed three to four times with PBS. Incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes; 1:500) was done for 1 hour at room temperature, followed by repeated washes using PBS. After subsequent staining of nuclei with 1 μ M DRAQ5 in PBS for 30 minutes, confocal images were obtained on a Zeiss LSM510 NLO laser-scanning microscope using single-line (488 nm) or multitrack sequential excitation (488 and 633 nm). Images were acquired and processed with Zeiss LSM Image Browser software.

Flow-cytometric analysis of CD30 expression

Expression of CD30 on the surface of mesothelioma cell lines was confirmed by FACS analysis, using a fluorescent dye-conjugated secondary antibody Alexa Fluor 488-conjugated anti-rabbit IgG (Molecular Probes; 1:500). Mesothelioma cells were collected by centrifugation, fixed with ethanol by incubating at least 30 minutes at room temperature. Centrifuged again and supernatant decanted off. Cells were permeabilized or nonpermeabilized with or without Triton X-100 (0.1% in PBS) for 10 minutes, briefly centrifuged and the resulting supernatant removed. The cells were suspended in FACS buffer with or without diluted primary (rabbit polyclonal anti-CD30 antibody, NBP1-72175, 1:100) or secondary (Alexa Fluor 488-conjugated anti-rabbit IgG, 1:500) antibodies followed by 30-minute incubation on ice. The stained cells were analyzed on a LSR II flow cytometer (BD Biosciences). FACS data were analyzed by using FlowJo software (TreeStar).

Cell proliferation analysis

Cell growth and viability were assessed in manually counted cells by either Trypan blue dye exclusion or the MTS assay, as described previously (15).

Cell-cycle analysis

Karpas 299, H2052, H2452, and H526 cells were seeded in 100 mm dishes and the next day treated with 0.05–60 μ g/mL of brentuximab vedotin for 48 hours. All attached and floating cells were washed with PBS and fixed in 0.125% formaldehyde followed by methanol. After incubation at –20°C, cells were washed

with PBS and incubated for 45 minutes at 4°C in 50 μ g/mL propidium iodide, 0.1% Nonidet P-40, 20 μ g/mL RNase A, and 0.1% sodium azide in PBS. Propidium iodide fluorescence was measured on an EPICS XL-MCL cytometer (Beckman Coulter).

IHC

The tissue microarray slides were obtained from the National Mesothelioma Virtual Bank (NMVB; <http://mesotissue.org/>). The slides were deparaffinized with xylene then transferred through graded ethanol to H₂O. Endogenous peroxidase activity was blocked by a 10-minute incubation in a 3% hydrogen peroxide solution. Antigen retrieval was performed by boiling the slides in a pressure cooker filled with a sodium citrate buffer (pH 6.0). After antigen retrieval, the slides were blocked using Background Sniper (Biocare, BS966M) for 20 minutes. The tissues were incubated with a mouse monoclonal anti-human CD30 antibody (Cell Marque/Clone Ber-H2) overnight at 4°C. Bound antibody was detected as described previously (16). The intensity of immunostaining for CD30 was scored visually by a thoracic pathologist and stratified into three staining groups based on staining intensity and percentage of tumor cells involved. Multiple cores for a single tumor sample were viewed to determine the score.

Statistical analysis

All the described experiments were done more than three times, and the data are presented as mean values \pm SEM. *P* values were determined by a *t* test using either Prism or SAS software (SAS Institute). *P* < 0.05 was considered statistically significant.

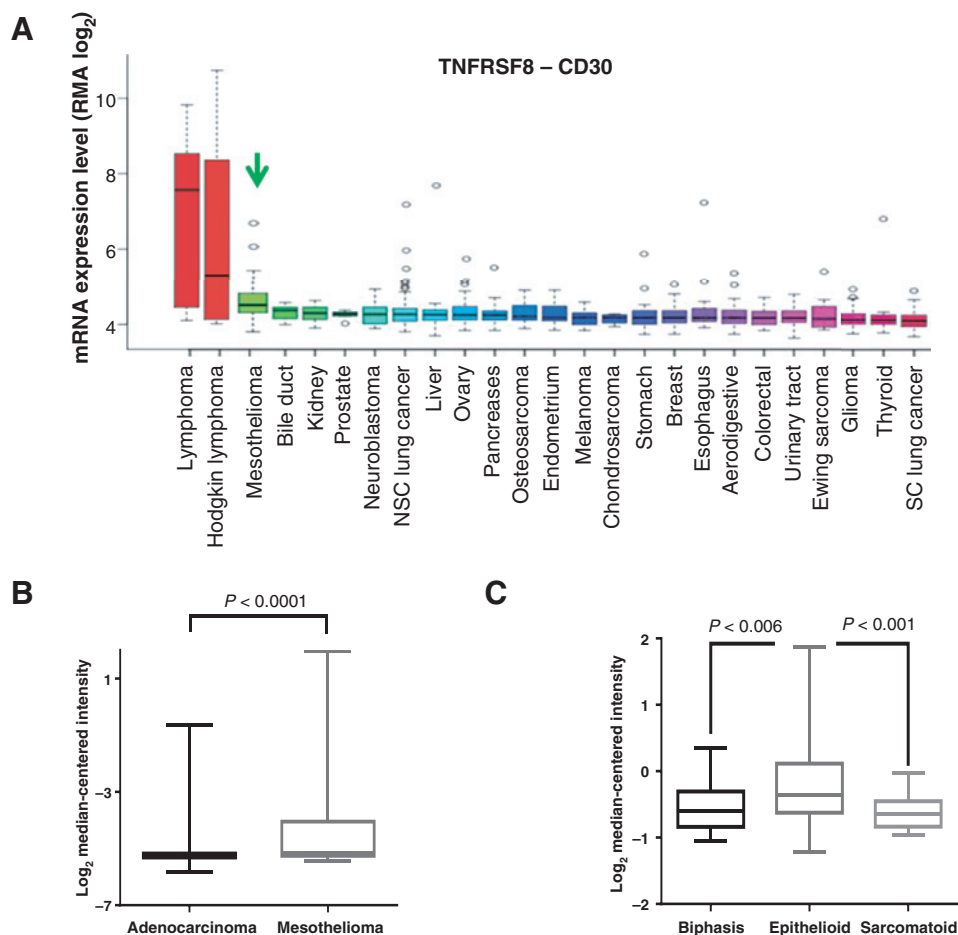
Results

CD30 mRNA expression is high in mesothelioma tissues

We analyzed the gene expression patterns of CD30 in all types of adult solid tumor cancer cell lines in the Cancer Cell Line Encyclopedia (CCLE) database (<http://www.broadinstitute.org/ccle/home>; ref. 17). When we compared cell lines derived from solid tumors, we found the highest CD30 expression levels in mesothelioma, excluding lymphomas (Fig. 1A). On the basis of these observations, we searched the OncoPrint database (<https://www.oncoprint.org/resource/login.html>) for expression in tumor tissues. A study by Gordon and colleagues (18) performed expression profiling in thoracic cancers. When we specifically looked at CD30 expression, we found that mesothelioma showed significantly higher expression of CD30 (*P* < 0.0001) compared with adenocarcinoma (Fig. 1B). A study by Lopez-Rios and colleagues (19) performed expression profiling in mesothelioma subtypes. When we specifically looked at CD30 expression in this dataset, we observed significantly higher CD30 expression in epithelioid compared with biphasic (*P* < 0.006) and sarcomatoid (*P* < 0.001) subtypes of mesothelioma tumors. Biphasic expression was not statistically different from sarcomatoid (*P* < 0.506; Fig. 1C).

CD30 protein is expressed in mesothelioma tumors

We obtained tissue microarrays from the NMVB to determine the level of CD30 staining in mesothelioma tumors by IHC. Descriptive statistics for the entire cohort of analyzed samples, as well as for CD30⁺ samples, are given in Table 1. The cohort demonstrated a typical distribution of histologic

**Figure 1.**

Mesothelioma shows high CD30 expression in public datasets. A, gene expression pattern for CD30 across solid tumor cancer cell lines in the CCLE database. B, whisker plots showing the log₂ median-centered intensity of CD30 expression in the Gordon lung dataset (18). Compared with adenocarcinoma, CD30 expression in mesothelioma tissue samples is significantly higher. C, whisker plots of the log₂ median-centered intensity of CD30 expression in the Lopez-Rios dataset (19) shows higher CD30 expression in epithelioid compared with biphasic and sarcomatoid.

subtypes. Overall, CD30⁺ expression was noted in 13 out of 83 total mesothelioma specimens and 12 of the CD30⁺ tumors demonstrated epithelial histology. The incidence of CD30 positivity was 4.6% in the biphasic and sarcomatoid group versus 19.7% for the epithelioid group, approaching significance ($P < 0.094$). Membrane-associated staining was evident in the highest scoring tumors, similar to that observed in the Hodgkin lymphoma positive control. Representative results of CD30 positivity are shown in Fig. 2 at three different magnifications. Diffuse cytoplasmic staining of variable intensity was observed in lower scoring mesothelioma tumors. The percentage of tumor cells stained positive varied greatly, from nearly the whole tumor core down to only 5% to 10% of the core, as

shown in the low-power images in Fig. 2, and generally decreased with scoring grade. One epithelioid core demonstrated 3+ and three demonstrated 2+ staining and the rest exhibited 1+ staining. The remaining CD30⁺ tumor was a high grade, biphasic metastatic tumor that demonstrated 1+ staining. We also looked for CD30 expression in three mesothelioma specimens obtained from our own hospital and found only one positive sample, although it demonstrated strong (3+) CD30 expression (data not shown). The mesothelioma cohort contained survival data on 63 patients; however, univariate analyses using both Kaplan–Meier ($P < 0.935$) and continuous measurement Cox regression models ($P < 0.82$) predicted no statistical significance of CD30 positivity on overall survival.

Table 1. CD30 positivity in mesothelioma cohort

	Whole cohort	CD30 ⁺	P
Histologic type			
Biphasic, sarcomatoid	22	1	0.094
Epithelioid	61	12	
Stage ^a			
1, 2, and 3	52	6	0.465
4	10	2	
Tumor grade ^a (epithelioid only)			
High	24	7	0.163
Intermediate and low	35	0	

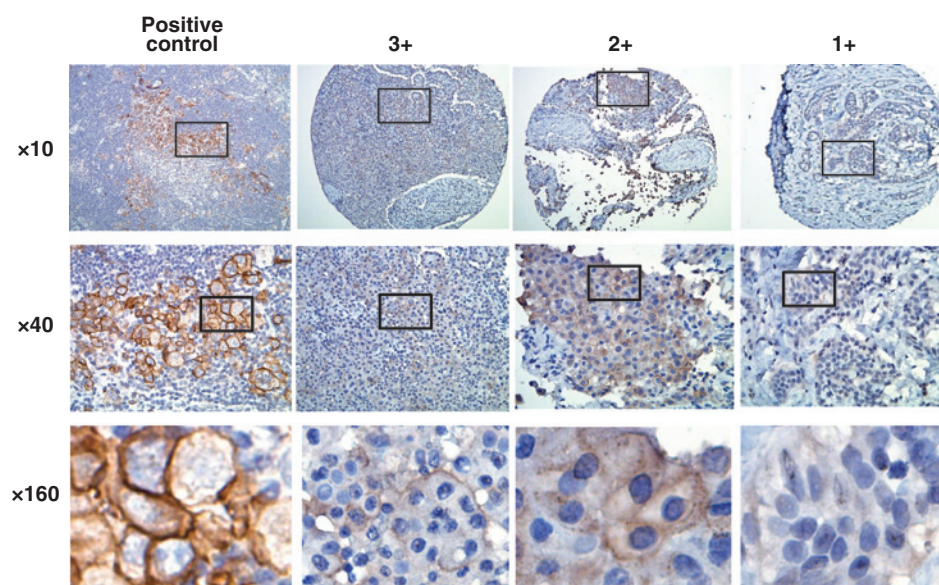
^aIncomplete staging and grading data available.

CD30 protein is expressed on the cell surface of mesothelioma cells

The IHC of CD30 in tumors clearly demonstrated a pattern of membrane-associated staining in some samples, which may represent an important feature in predicting sensitivity to brentuximab vedotin. We therefore sought to validate this finding in mesothelioma cell lines. We obtained all the available mesothelioma cell lines from the ATCC and determined the subcellular localization of CD30 by confocal microscopy. Data from the CCLE indicated that all four mesothelioma cells expressed CD30 mRNA. Figure 3A shows that CD30 staining outlines the shape of

Figure 2.

Mesothelioma tumor specimens demonstrate high CD30 expression. CD30 expression was examined by IHC with an anti-BerH2 antibody used for the clinical diagnosis of Hodgkin disease. The positive control is Hodgkin lymphoma. Rectangles show the area magnified in the image below. The 3+ core was scored higher than the 2+ core due to a greater area of positivity, not because of intensity.



two mesothelioma cell lines, H2052 and H2452, consistent with CD30 residing on the cell membrane. Further examination of other focal planes confirmed this finding and detected little or no staining of cytoplasm. Similar results were obtained for 211H and H28 cells (data not shown). These results indicated that in mesothelioma cell lines, CD30 resides on the cellular membrane.

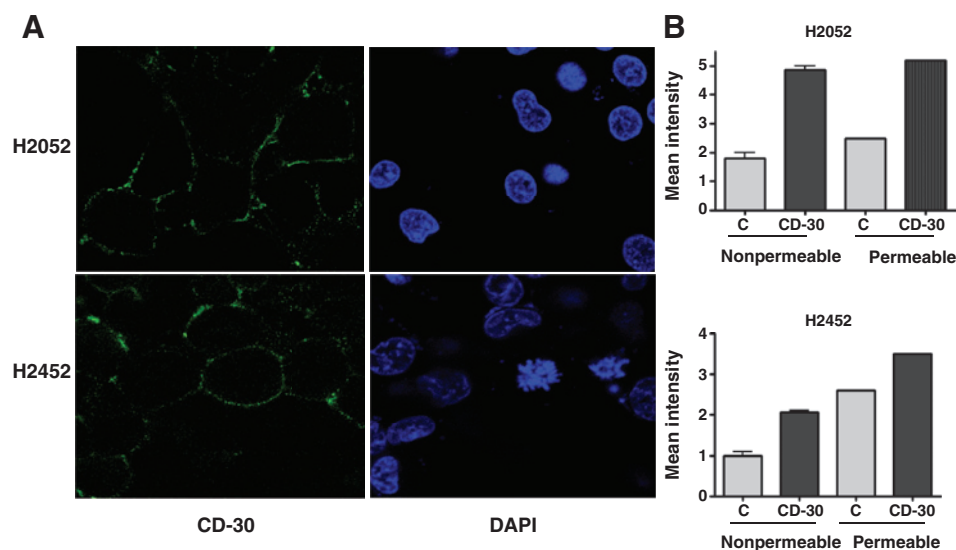
To determine whether CD30 is expressed on the cell surface, H2052 and H2452 cells were fixed with ethanol, before being stained with an anti-CD30 antibody, and then analyzed by flow cytometry (Fig. 3B). Both cell lines were found to be positive for CD30 when intact (nonpermeabilized). However, when cells were permeabilized with Triton X-100 before staining to reveal intracellular CD30, variable amounts of additional CD30 were detected. Taken together, these results indicate that a significant amount of cellular CD30 antigen is located on the cell-surface of mesothelioma cells.

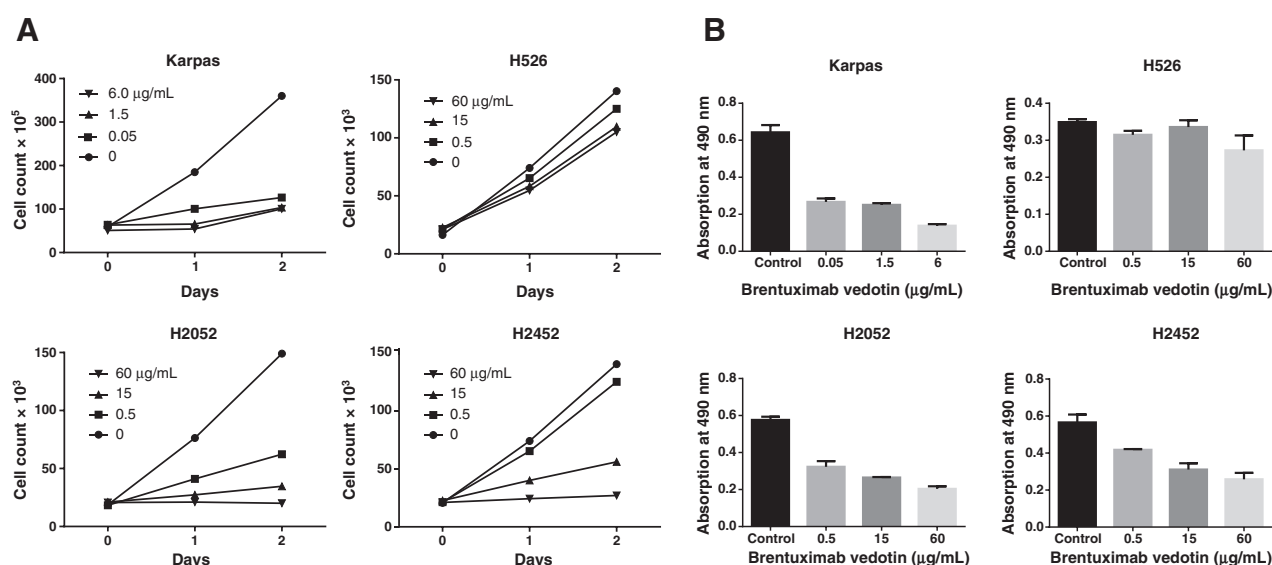
Brentuximab vedotin decreases cell growth and viability in mesothelioma cells

We hypothesize that the expression of CD30 on the cell surface should sensitize mesothelioma cells to brentuximab vedotin. We therefore investigated the effect of this antibody–drug conjugate on cell growth in mesothelioma cell lines. We selected Karpas 299, a CD30⁺ lymphoma cell line, as a positive control and H526, a small cell lung cancer cell line, as a negative control for these experiments. Cell growth determination by viable cell count demonstrated that Karpas cells were sensitive to brentuximab vedotin in a dose-dependent manner after 48 hours of treatment, as expected, whereas H526 cells were largely resistant, indicating that most of the toxic drug remained conjugated to the CD30 antibody (Fig. 4A). The two mesothelioma cell lines tested, H2052 and H2452, were both sensitive to the antibody–drug conjugate, with H2052 cells demonstrating more sensitivity than H2452 cells, although both were less sensitive than Karpas cells.

Figure 3.

Analysis of CD30 expression in mesothelioma cell lines. A, confocal analysis of mesothelioma cell lines. The DAPI stain identifies the nucleus. B, mesothelioma CD30 expression by FACS analysis. In these experiments, control is staining in the absence of primary antibody.



**Figure 4.**

Effect of brentuximab vedotin treatment on cell growth and viability: A, mesothelioma cell lines H2052, H2452, and positive control Karpas 299 treated with brentuximab vedotin and cells were counted on day 1 and 2. B, MTS assay of cell proliferation.

We further investigated the effect of brentuximab vedotin on mesothelioma cell viability using the MTS assay. Treatment of H2052 and H2452 cells for 48 hours induced a dose-dependent decrease in cell viability compared with untreated control cells (Fig. 4B). As expected, the Karpas cells demonstrated greater sensitivity than mesothelioma cells, whereas the H526 cells were resistant.

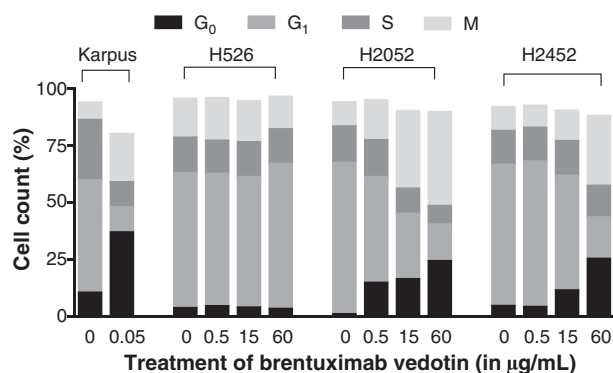
To confirm that brentuximab vedotin induced cell death in mesothelioma cells, we performed cell-cycle analysis by flow cytometry. As shown in Fig. 5, brentuximab vedotin greatly increased the proportion of Karpas cells in the G_0 (sub- G_1) phase at the lowest dose after 48 hours of incubation. This effect was not observed with H526 cells at any dose. Importantly, the two mesothelioma cell lines tested, H2052 and H2452, also demonstrated a dose-dependent increase in the proportion of cells in the sub- G_1 phase in response to brentuximab vedotin treatment, with H2052 cells again being more

sensitive to drug than H2452 cells. The increased proportion of mesothelioma cells in sub- G_1 phase was accompanied by a parallel increase in cells in the G_2 -M phase. This is consistent with the anti-microtubule activity of the conjugated drug in brentuximab vedotin, MMAE, to produce mitotic arrest and G_2 -M blockade. Taken together, these results demonstrate that brentuximab vedotin treatment has an inhibitory effect on cell growth and survival in mesothelioma cells.

Discussion

The goal of this study was to determine the therapeutic potential of targeting CD30 with brentuximab vedotin in mesothelioma. We looked for CD30 expression in the largest cohort of mesothelioma tumors studied to date and found that only 15.6% (13 of 83 tumors) were positive for CD30 expression using an antisera routinely used in the diagnosis of Hodgkin lymphoma. There was a trend toward CD30 expression in mesothelioma tumors with epithelioid histology and high grade. Previous studies of CD30 expression in mesothelioma had noted expression in a single index case (4), two of five tumors (20) and two of eight tumors and 16 of 28 pleural and peritoneal effusions (5).

Although CD30 expression was not prognostic for overall survival in our study, of greater importance was whether or not CD30 expression could predict sensitivity to the antibody-drug conjugate targeting CD30, brentuximab vedotin. We used mesothelioma cell lines as a model system to test this hypothesis. Our results demonstrated that CD30 was expressed on the cell surface of mesothelioma cells using two complementary methods, confocal microscopy and FACS analysis. We then demonstrated that brentuximab vedotin decreased cell growth and viability in CD30-positive mesothelioma cells by three different methods. It is important to highlight three crucial points in these experiments. First, the doses of brentuximab vedotin used in our study, 0.5, 15, and 60 µg/mL, were within the range of drug clinically achievable. That is, the typical C_{max} and AUC for brentuximab

**Figure 5.**

Effect of brentuximab vedotin treatment on the cell cycle. Results demonstrate a dose response of mesothelioma cells to brentuximab vedotin treatment after 48 hours. The percentage of cells in the G_0 , G_1 , S, and M phase is shown.

vedotin after a single 1.8 mg/kg dose were approximately 32 and 80 $\mu\text{g/mL/day}$, respectively, in a phase I study (Acedris insert). Second, the loss of mesothelioma cell viability after brentuximab vedotin treatment was associated with an increase in the proportion of cells in G₂-M phase, as expected because the antibody-conjugated toxin MMAE is a microtubule inhibitor. Third, there was no effect of brentuximab vedotin on a small cell lung cancer cell line, H526, suggesting therapeutic specificity.

It is unclear, however, to what extent our demonstration of brentuximab vedotin sensitivity *in vitro* using cultured mesothelioma cells is translatable to clinical drug sensitivity in mesothelioma patients. In this regard, a recent interim report from a phase II study by Jalal and colleagues on brentuximab vedotin in patients with malignant mesothelioma reported that 34% were positive for CD30 expression and that five positive responses were obtained to drug treatment out of 21 evaluable CD30⁺ patients (21). Although we obtained a lower percentage of CD30⁺ tumors (15.6%), likely attributable to scoring methods, the interim results of this phase II study confirm our hypothesis that a subgroup of malignant mesothelioma patients will respond to brentuximab.

It is clear from our study that mesothelioma tumor specimens demonstrated a range of CD30 expression, both in terms of their staining intensity and percentage of tumor cells involved. However, there was clearly a subset of high-scoring tumors that demonstrated obvious membrane-associated staining. It is intriguing to think that these tumors may demonstrate the greatest sensitivity to brentuximab vedotin. It has been suggested, however, that the MMAE toxin is freely diffusible into the surrounding microenvironment once it is internalized and cleaved from CD30 antibody in target CD30⁺ lymphoma cells, as evidenced by the sometimes-low occupancy (3%) of CD30-binding sites in lymphoma cells from highly responsive patients treated with brentuximab vedotin (22). Thus, the efficacy of brentuximab vedotin in mesothelioma tumors may not simply correlate with CD30

positivity. The answer to these questions awaits more detailed analysis of CD30 expression in clinical trials of brentuximab vedotin in malignant mesothelioma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Dabir, A. Kresak, G. Wildey, A. Dowlati
Development of methodology: S. Dabir, A. Kresak, A. Dowlati
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Kresak, M. Yang, A. Dowlati
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Dabir, A. Kresak, P. Fu, G. Wildey, A. Dowlati
Writing, review, and/or revision of the manuscript: S. Dabir, A. Kresak, G. Wildey, A. Dowlati
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Kresak, M. Yang, G. Wildey, A. Dowlati
Study supervision: G. Wildey, A. Dowlati

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