Constitutive activation of metalloproteinase ADAM10 in mantle cell lymphoma promotes cell growth and activates the TNFα/NFκB pathway

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ABSTRACT

One of the main functions of ADAM10, a disintegrin and metalloproteinase, is to regulate the bioavailability of adhesion molecules and ligands to various cellular signaling receptors. Constitutive activation of ADAM10 has been implicated in the pathogenesis of several types of solid tumors. In this study, we found that mantle cell lymphoma (MCL) cell lines and all 12 patient samples examined expressed the active/mature form of ADAM10. In contrast, peripheral blood mononuclear cells from healthy donors (n=5) were negative. By immunohistochemistry, ADAM10 was readily detectable in 20 of 23 (87%) MCL tumors but absent in 5 reactive tonsils. Knockdown of ADAM10 using siRNA in MCL cells significantly induced growth inhibition and cell-cycle arrest, and these changes correlated with a downregulation of cyclin D1, upregulation of p21\textsuperscript{waf1}, and significant reductions in the TNF\textgreek{a} production/transcriptional activity of NF\textgreek{k}Bp65. Addition of recombinant ADAM10 to MCL cells led to the opposite biological effects. Lastly, downregulation of ADAM10 using siRNA enhanced the growth-suppressing effects mediated by proteasome inhibitors MG132 and bortezomib. To conclude, constitutive activation of ADAM10 contributes to the growth of MCL. Inhibition of ADAM10 may be a useful strategy to enhance the response of MCL to other therapeutic agents.
INTRODUCTION

A disintegrin and metalloproteinase 10 (ADAM10), a member of the ADAM family of metalloproteinases, was discovered in the protein extract of brain myelin membranes and subsequently found to be a homologue of the Drosophila kuzbanian (kuz) gene. ADAM10 is secreted as a precursor protein, and it consists of multiple functional domains including a prodomain, catalytic domain, cysteine-rich domain, transmembrane domain and cytoplasmic domain. To become the active/mature form, the precursor ADAM10 protein needs to be cleaved by proprotein convertase 7 and furin, both of which remove the prodomain of ADAM10. ADAM10 is biologically important, since ADAM10 knockout mice die on day 9 of embryogenesis due to multiple abnormalities in the nervous and cardiovascular systems. The key biological function of ADAM10 appears to be attributed to its enzymatic activity as a metalloproteinase. Specifically, ADAM10 is involved in the intra-membrane proteolysis process, whereby it mediates ectodomain shedding of various membrane bound receptors, adhesion molecules, growth factors and cytokines. For instance, ADAM10 is involved in the regulation of the shedding of Notch, HER-2, CD44, IL-6 receptor, amyloid precursor protein and cadherins. Directly relevant to our study, ADAM10 was recently found to be one of the enzymes responsible for cleaving tumor necrosis factor alpha (TNFα) and releasing its active form. Furthermore, it has been reported that ADAM10 is important for the development of the marginal zone B-cells. By regulating the bioavailability of ligands to various cellular signaling receptors, ADAM10 modulates the activation status of various cellular signaling pathways that have an impact on various cellular responses such as proliferation and migration. ADAM10 has been shown to be
constitutively active in a number of solid tumors, and this biochemical defect is implicated in the pathogenesis of these tumors. For instance, xenografting of colorectal cancer cells with enforced expression of ADAM10 in nude mice induced the formation of liver metastasis compared to the negative control cells, and this effect can be attributed to ADAM10-mediated cleavage and release of L1-CAM, a cell adhesion molecule. In another study, ADAM10 expression in colorectal cancer patient samples detectable by immunohistochemistry was found to correlate with a higher clinical stage. Using immunohistochemistry, it was also found that ADAM10 is overexpressed in squamous cell carcinomas of the oral cavity, as compared to the benign epithelial cells; knockdown of ADAM10 expression using siRNA in the cell lines derived from these tumors was shown to induce a significant decrease in cell growth. Similar findings were made in pancreatic cancer, where inhibition of ADAM10 expression in pancreatic carcinoma cell lines resulted in a significant decrease in invasiveness and migration. Lastly, ADAM10-mediated cleavage of N-cadherin was found to regulate the migratory properties of glioblastoma cells. While the pathogenetic role of ADAM10 has been well documented in solid tumors, its role in hematologic malignancies is largely unknown. To our knowledge, there is only one published study that performed a survey of the expression of various ADAM family members in benign and malignant hematopoietic cells using RT-PCR, and the authors reported the expression of the ADAM10 mRNA in myeloma, erythroleukemia and a subset of lymphoma cell lines. Since only RT-PCR was used in this particular study, whether ADAM10 is in its active form could not be assessed; the biological significance of ADAM10 in these malignancies also was not examined.
Mantle cell lymphoma (MCL) is a specific subtype of aggressive B-cell lymphoma recognized by the World Health Organization Classification Scheme. The genetic hallmark of this disease is the recurrent chromosomal abnormality, \( t(11;14)(q13;q32) \), which brings the \( cyclin\ D1(CCN\ D1) \) gene under the influence of the enhancer of the immunoglobulin heavy chain (\( IgH \)) gene, leading to cyclin D1 overexpression.

Although it has been shown that cyclin D1 overexpression is not sufficient for the induction of lymphoma in animal models, this abnormality is considered to be the primary oncogenic event in MCL. Large-scale cDNA microarray studies using frozen MCL tumors have revealed a relatively large number of biochemical abnormalities in MCL, with these defects are frequently implicated in the regulation of apoptosis, survival and DNA damage. An increasing number of cellular signaling pathways also have been found to be abnormal. For instance, NF\( \kappa \)B has been reported to be constitutively active in MCL, and this biochemical defect is biologically important to the pathogenesis of MCL. The activation of NF\( \kappa \)B in MCL can be partly attributed to TNF\( \alpha \), the bioavailability of which has been shown to be regulated by ADAM10, as mentioned above.

In this report, we described for the first time that ADAM10 is constitutively activated in MCL cell lines and tumors. In view of the importance of TNF\( \alpha \) in the biology of MCL, and the previous report that ADAM10 can increase the bioavailability of TNF\( \alpha \), we hypothesize that ADAM10 may be important in the pathogenesis of MCL.
MATERIALS AND METHODS

1. Cell lines and tissue culture

The characteristics of the three MCL cell lines, Jeko-1, Mino, and SP53, have been previously described. All 3 cell lines are negative for the Epstein-Barr virus nuclear antigen. They were grown at 37°C and 5% CO₂ and maintained in RPMI medium (Sigma-Aldrich, St. Louis, MO). The culture media were enriched with 10% fetal bovine serum (Life Technologies, Carlsbad, CA). Ficoll-Paque (GE Health care, Quebec, Canada) was used to isolate peripheral blood mononuclear cells (PBMC) from healthy donors (n=5) and leukemic MCL patients (n=3). OCT-embedded frozen tumors from classical MCL (n=9), chronic lymphocytic leukemias (CLL, n=5), follicular lymphomas (FL, n=4), diffuse large B-cell lymphomas (DLBCL, n=4) and marginal zone lymphomas (MZL, n=4) were also included for comparison. The use of these patient samples has been reviewed and approved by our Institutional Ethics Committee.

2. Subcellular protein fractionation, Western blots and antibodies

For subcellular protein fractionation, we employed a kit purchased from Active Motif (Carlsbad, CA) and followed the manufacturer’s instructions. Preparation of cell lysates for Western blots was done as previously described. Antibodies employed in this study included those reactive with ADAM10, phospho-NFκBp65 (Chemicon, Temecula, CA), NFκBp65, cyclin D1, tubulin, HDAC and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), p21Waf1, p27Kip1, p15, p16, cleaved PARP and cleaved caspases 3, 7 and 9 (Cell Signaling Technology, Danvers, MA).
3. Short interfering RNA (siRNA)

Two siRNA species for ADAM10 from 2 different commercial sources were used in this study. siRNA #1 was a pool of 4 siRNA species from Dharmacon (Lafayette, CO) and siRNA #2 was from Invitrogen (Burlington, Ontario, Canada). Scrambled siRNA was purchased from Dharmacon. Transfection of siRNA was carried out using an electrosquare electroporator, BTX ECM 800 (225V, 8.5ms, 3 pulses) (Holliston, MA). The concentration of siRNA used was 200 pmol/1x10^6 cells, and cells were harvested at 48 hours after transfection. The ADAM10 protein levels were assessed using Western blot to evaluate the efficiency of inhibition.

4. Cell viability

A total of 100,000 cells suspended in 1 mL of culture medium were plated in triplicate. To assess cell viability, trypan blue exclusion assay (Sigma-Aldrich) was performed every 24 hours for up to 3 days following transfection of ADAM10 siRNA or scrambled siRNA. MTS assay was also performed during this time frame and the manufacturer’s instructions were followed (Promega, Madison, WI). The measurements were obtained at a wavelength of 450 nM using a Bio-Rad Micro plate Reader (Bio-Rad, Hercules, CA). The absorbance values were normalized to the wells with media only using the microplate Manager 5.2.1 software (Bio-Rad). All experiments were performed in triplicates.

5. Recombinant ADAM10, TNFα assay, bortezomib and MG132
Human recombinant ADAM10 was purchased from BD biosciences (Pharmingen, San Diego, CA) and it corresponds to the segment spanning from Thr214 to Glu672, which represents the active/mature form of ADAM10. It was added at a concentration of 100 ng/ml to 1x10^6 cells; after 24 hours, cell lysates were prepared and cell count was done. TNFα secretion was monitored using a commercially available ELISA kit (R&D Systems Inc., Minneapolis, MN). Aliquots of the culture medium from MCL cell lines collected 48 hours after transfection with either scrambled siRNA or ADAM10 siRNA were centrifuged at 15,000 g, and the supernatant was assayed for the TNFα levels as per manufacturer's protocol. Bortezomib (LC laboratories, Woburn, MA), a proteasome inhibitor was added at a concentration of 5 nM to MCL cells 24 hours after siRNA transfection, and cell count using trypan blue exclusion assay was performed 48 hours after the initiation of the experiment. Another proteasome inhibitor MG132 (Calbiochem, EMD Biosciences, Darmstadt, Germany) was added at a concentration of 1 μM to MCL cells 24 hours after siRNA transfection, and cell count using trypan blue exclusion assay was performed 48 hours after the initiation of the experiment.

6. NFκB transcriptional activity

To assess the transcriptional activity of NFκB in MCL cell lines, we employed the NFκB responsive firefly luciferase reporter plasmid and renilla reporter plasmid (Promega). After 48 hours of transfection of the reporter plasmid (with the renilla luciferase plasmid as an internal control) together with either scrambled siRNA or ADAM10 siRNA, MCL cells were harvested and cell extracts were prepared using a lysis buffer purchased
from Promega. The firefly luciferase activity and renilla luciferase activity were assessed using the dual luciferase reagent (Promega).

7. Cell cycle analysis by flow cytometry
Cells transfected with either scrambled siRNA or ADAM10 siRNA were fixed with ice-cold 70% ethanol 24 hours after gene transfection. These cells were then subjected to RNase treatment and propidium iodide (PI) staining. DNA content was determined using a FACSCalibur flow cytometer (BD Biosciences). Data acquisition was gated to exclude cell doublets and the cell-cycle phase distribution was determined using the CellQuest program (20,000 events were counted).

8. Assessment of cyclin D1 expression using quantitative RT-PCR
The expression of *cyclin D1* in MCL cells treated with ADAM10 siRNA was assessed using quantitative RT-PCR (qRT-PCR). The assay was performed using the Applied Biosystems 7900 HT, and the SYBR® GreenER™ qPCR SuperMix from Invitrogen. The primer sequence for *ADAM10* was as follows: forward 5' - AGCAACATCTGGGGACAAAC -3'; reverse 5' - CTTCCCTCTGGTTGATTTGC -3'. The primer sequence for *cyclin D1* was as follows: forward 5' - CAAATGGAGCTGCTCCTGGTG -3'; reverse 5' - TGGCACCAGCCTCGGCATTTC -3'. 34 Triplicate experiments were performed and the statistical significance of the differences was assessed using Student *t*-test.

9. Immunohistochemistry and archival MCL tumors
Formalin-fixed, paraffin-embedded tumors from a cohort of 23 patients with classical MCL were retrieved from the files at the Cross Cancer Institute (Edmonton, Alberta, Canada). All MCL primary tumors were diagnosed at the Cross Cancer Institute and the diagnostic criteria were based on those described in the World Health Organization Classification Scheme. All cases were confirmed to express cyclin D1 by immunohistochemistry. The use of these tissues has been approved by our Institutional Ethics Committee. Immunohistochemistry was performed using standard techniques. Briefly, formalin-fixed, paraffin-embedded tissue sections of 4 μM thickness were deparaffinized and hydrated. Heat-induced epitope retrieval was performed in a pressure cooker using citrate buffer (pH=6) in a microwave. After antigen retrieval, tissue sections were incubated with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. Tissue sections were then incubated with anti-ADAM10 antibody (1:200) (same antibody used in western blots) overnight in a humidified chamber at 4°C. Immunostaining was visualized with a labeled streptavidin-biotin (LSAB) method using 3,3'-diaminobenzidine as a chromogen (Dako Canada Inc., Mississauga, Ontario, Canada) and counter-stained with hematoxylin. Colon carcinoma case served as the positive control, whereas the lymphoid cells of mantle zone in benign tonsils served as the negative control.

10. Statistical analysis

Data are expressed as mean +/- standard derivation. Unless stated otherwise, statistical significance was determined using two-tailed Student's t-test and statistical significance was achieved when the p value is <0.05. In experiments where we needed to determine
the statistical significance between more than two groups analysis of variance (ANOVA) test was applied.

RESULTS

1. The active/mature form of ADAM10 is expressed in MCL cells and other B-cell lineage malignancies

The expression of ADAM10 in 3 MCL cell lines was assessed using western blots, and the results are illustrated in figure 1A. While the precursor form of ADAM10 at 98 kDa was highly expressed in all 3 cell lines examined, the expression levels of the active/mature form of ADAM10 at 72 kDa were variable among these 3 cell lines. Specifically, although the active/mature form of ADAM10 was readily detectable in Jeko-1 and Mino cells, it was barely detectable in SP53 cells. MCF-7, a breast cancer cell line previously reported to have a high level of active/mature form of ADAM10, served as the positive control. In contrast to MCL cells, PBMC from five healthy donors expressed no detectable active/mature form of ADAM10 at 72 kDa, although the precursor form was readily detectable in all cases. The results from 3 of these 5 healthy individuals are illustrated in figure 1A and 1B.

Western blot analysis was performed using 9 frozen MCL tumor samples (patient #1-9) and 3 fresh leukemic MCL blood samples (patient #10-12). The active/mature form of ADAM10 was readily detectable in all patient samples, although the level was relatively low in patient #7 (figure 1B). Again, PBMC from healthy donor #2 and #3 had no convincing band at 72 kDa. Of note, in contrast with the MCL cell lines (figure 1A); most
MCL patient samples expressed the active/mature form of ADAM10 at a higher level than its precursor form. We also analyzed the expression of both the precursor and active/mature forms of ADAM10 in frozen tissues derived from other B-cell lineage malignancies (5 CLL cases, 4 DLBCL cases, 4 FL cases and 4 MZL cases). As shown in supplementary figure 1, the expression of ADAM10 was readily detectable in all of these B-cell neoplasms, with a pattern similar to that seen in MCL patient samples.

We then performed immunohistochemistry applied to 23 MCL formalin-fixed/paraffin-embedded tumor samples and 5 cases of benign reactive tonsils. We found that the mantle zones in benign reactive tonsils were negative for ADAM10, whereas the germinal centers showed only faint cytoplasmic staining (figure 1C). The ADAM10 immunostaining in MCL was generally homogenous within the same tumor, and we considered a MCL tumor to be positive when the majority of the tumor cells showed a staining intensity higher than that of germinal center cells. With this criterion, ADAM10 was assessed positive in 20 of 23 (87%) cases. On higher magnification, the immunostaining was predominantly cytoplasmic in all cases, while a small subset of cases also showed some degree of nuclear staining (figure 1C). Of note, 5 of these MCL cases used for immunohistochemical studies were also included in the experiment illustrated in figure 1B (western blot studies); all these 5 cases were positive by immunohistochemistry and carried the active form of ADAM10 by western blots.

2. ADAM10 promotes cell growth in MCL cells
To assess the functional importance of ADAM10 in MCL cells, we evaluated if siRNA knockdown of ADAM10 has any impact on the growth of MCL cells. As shown in figure 2A, transfection of siRNA into Jeko-1 and Mino resulted in a reduction of both the precursor and the active/mature form of ADAM10. With the knockdown of ADAM10, the cell growth (assessed by the trypan blue exclusion assay) was significantly decreased, as compared to cells transfected with scrambled siRNA (p=0.01 and 0.007 for Mino and Jeko-1, respectively) three days after transfection (figure 2B). The decrease in the viable cell count was not associated with any substantial increase in trypan blue-positive dead cells. Similar experiments were performed using the MTS assay, and we found comparable results (p<0.0001 and p=0.01 for Mino and Jeko-1, respectively) (figure 2C). To further strengthen the conclusion that ADAM10 promotes cell growth in MCL cells, we tested how MCL cells may respond to recombinant ADAM10. SP53 and Mino cells were used for this experiment, since both cell lines had a lower level of active ADAM10 than Jeko-1 cells, and thus, they were expected to show more dramatic response to recombinant ADAM10. As shown in figure 2D, the addition of recombinant ADAM10 induced a significant increase in cell growth (p=0.008 and 0.01, respectively).

3. Downregulation of ADAM10 induces cell cycle arrest but not apoptosis

To characterize the mechanism by which ADAM10 promotes cell growth in MCL, we performed cell cycle analysis. Transfection of ADAM10 siRNA into Mino and Jeko-1 cells induced a significant G₀/₁ arrest and reduced the proportion of cells in the S phase, as compared to cells transfected with scrambled siRNA (p=0.02 and 0.05 for Mino and Jeko-1, respectively). Of note, no appreciable increase in the proportion of cells in the...
subG₀/₁ phase was found. All experiments were performed in triplicates and representative results are shown in figure 3. To further exclude the occurrence of apoptosis, we performed western blots and found no detectable cleavage of caspases 3, 7, 9 and PARP (supplementary figure 2). Cells treated with MG132 served as positive controls.

4. ADAM10 regulates the cyclin D1 expression level in MCL cells

Since cyclin D1 overexpression is believed to play an important pathogenetic role in MCL, we assessed if ADAM10 mediates any effects on the expression of cyclin D1 in MCL cell lines. As shown in figure 4A, downregulation of ADAM10 using siRNA appreciably reduced the cyclin D1 protein expression level in Jeko-1 and Mino cells. This decrease in the cyclin D1 protein level correlated well with our observations that the cyclin D1 mRNA levels were significantly reduced 24 hours after the treatment of ADAM10 siRNA (figure 4B). Specifically, the cyclin D1 mRNA levels detected by quantitative RT-PCR were reduced by 21% and 35% in Jeko-1 and Mino cells, respectively. Opposite effects were observed when recombinant ADAM10 (100 ng/ml) was added to SP53 cells (not shown). Using subcellular fractionation, we found that the increase in the cyclin D1 protein expression level was largely attributed to an increase in cyclin D1 in the nuclear fraction (figure 4C). We also examined if downregulation of ADAM10 using siRNA modulates a number of other cell-cycle regulators including p21^{Waf1}, p27^{Kip1}, p15 and p16; we found that there was an upregulation of p21^{Waf1}, an inhibitor of cyclin-dependent kinase 4 (figure 4A); the other three proteins showed no appreciable change (not shown).
5. ADAM10 activates the TNFα/NFκB axis

In view of the previous literature that ADAM10 regulates the production of TNFα in murine cell lines and immortalized human cell lines, \(^{11,13}\) as well as the importance of the TNFα in the biology of MCL \(^{29-31}\), we hypothesized that ADAM10 may promote the growth of MCL via upregulation of TNFα. In keeping with this concept, we found that downregulation of ADAM10 using siRNA in Jeko-1 cells resulted in a significant decrease in the levels of TNFα present in the culture media (\(p=0.001\)) (figure 5A). Since TNFα is an activator of NFκB \(^{36}\), a signaling protein also strongly implicated in the pathogenesis of MCL \(^{29}\), we then assessed the relationship between ADAM10 and this signaling protein. We performed subcellular fractionation experiments in which we found a correlation among the expression levels of the phosphorylated NFκB (active form), total NFκBp65 and ADAM10 in 3 MCL cell lines (supplementary figure 3). Specifically, we found that Jeko-1 cells, which have a relatively high level of the active/mature form of ADAM10, carried a higher level of phosphorylated NFκBp65 than SP53 cells, which have a much lower level of the active/mature form of ADAM10. As shown in figure 5B, there was a significant downregulation in both the phosphorylated and total NFκBp65 level in Mino and Jeko-1 cells after ADAM10 siRNA treatment. Using subcellular fractionation, we found that the protein level of NFκBp65 in the nuclear fractions was decreased, more so than that in the cytoplasmic fractions (figure 5C). Lastly, we assessed the transcriptional activity of NFκB using a commercially available reporter construct. As shown in figure 5D, there was significant downregulation of NFκB transcriptional activity following ADAM10 downregulation in Mino cells. In contrast,
addition of human recombinant ADAM10 (100 ng/mL) resulted in a significant increase in the NFκB transcriptional activity in these cells (figure 5E).

6. ADAM10 inhibition enhanced the growth suppressing effect of the proteasome inhibitors MG132 and bortezomib

Proteasome inhibitors have been shown to induce apoptosis and cell-cycle arrest in MCL cells, and these effects are believed to be mediated via an inhibition of the NFκB signaling pathway. Since we have shown that ADAM10 mediates its effects via the NFκB pathway, we hypothesized that ADAM10 inhibition may enhance the growth-suppressing effects of proteasome inhibitors such as MG132 in MCL cells. In our initial experiment, we determined that the inhibitory concentration at 50% (i.e. IC50) for MG132 was in the range of 1 μM for Jeko-1 cells (data not shown). Using this concentration of MG132, we found that Jeko-1 cells transfected with ADAM10 siRNA had a significantly more reduction in the number of viable cells, as compared to cells transfected with the scrambled siRNA (p< 0.0001, ANOVA) (figure 6A). We also performed similar experiments using bortezomib, a proteasome inhibitor currently used in various clinical trials for the treatment of MCL. We used 5 nM for this experiment, since cell growth inhibition was approximately 15% at this drug concentration. As shown in figure 6B, the combination of bortezomib and ADAM10 siRNA resulted in a significant reduction in number of viable cells, as compared to the use of bortezomib or ADAM10 siRNA alone (p< 0.0001, ANOVA)
DISCUSSION

ADAM10 has recently been implicated in the pathogenesis of several types of malignant solid tumors such as those arising from colon, pancreas, ovary, uterus and the oral cavity. For the first time, we have demonstrated that constitutive activation of ADAM10 is a highly frequent finding in MCL. Our findings also suggest that ADAM10 is biologically significant in MCL. Specifically, ADAM10 promotes cell-cycle progression and increased cell growth, which are associated with modulations of two important cell-cycle regulators (cyclin D1 and p21^{raf1}) and activation of the TNF{alpha}/NFkB signaling pathway. While MCL is the focus of this study, we found evidence of constitutive activation of ADAM10 in other types of B-cell non-Hodgkin lymphomas, although the functional significance of ADAM10 in these tumors needs to be further examined.

Regarding the association between ADAM10 and TNF{alpha}, we would like to point that, while ADAM10 have been reported previously to be an important sheddase for TNF{alpha}, all of these studies were done using murine fibroblasts or other immortalized human cell lines such as the human kidney cell line 293A and human articular chondrocytes. In this study, inhibition of ADAM10 expression with the use of siRNA led to downregulation of TNF{alpha} secretion. To our knowledge, this is the first study directly linking ADAM10 and TNF{alpha} in a human cancer model. Furthermore, this is also the first report describing the link between ADAM10 and NFkB. The pathogenetic importance of NFkB in MCL has been previously reported. Specifically, NFkB appears to confer anti-apoptotic signal in MCL, as blockade of this signaling protein in MCL cells induces downregulation of several anti-apoptotic proteins such as bcl-2 and bcl-xL. The link
between TNFα and NFκB in MCL also has been previously established in one of our previous studies.\textsuperscript{31} Taken together, it appears that ADAM10 upregulates the autocrine production of TNFα, thereby activating the NFκB signaling pathway. Interestingly, although pharmacologic inhibition of NFκB in MCL has been previously shown to result in significant apoptosis in MCL cells\textsuperscript{29,31}, we did not observe any detectable evidence of apoptosis as a result of ADAM10 knockdown using siRNA. This may be due to the fact that residual NFκB activity is sufficient to prevent the activation of the apoptotic pathway.

In the present study, we have shown that ADAM10 is frequently active in MCL cell lines and tumors, but not in PBMC from healthy donors. Interestingly, in the vast majority of the patient samples (n=12), the active/mature form of ADAM10 was expressed at a higher level than the precursor form of ADAM10. This pattern is in contrast with that of MCL cell lines, in which the precursor form of ADAM10 was expressed at a higher level than the active/mature form of ADAM10. While we cannot provide definitive explanations regarding this discrepancy between cell lines and tumors, we have considered the possibility that the tumor microenvironment may be involved in the regulation of ADAM10.

Our immunohistochemical data revealed that ADAM10 was readily detectable in most MCL tumors examined. This is in contrast with benign mantle zones, which did not show detectable ADAM10 immunoreactivity. These findings suggest that ADAM10 is 'over-expressed' in MCL cells, as compared to benign mantle zone cells. Of note, we
employed a commercially available, anti-ADAM10 antibody that recognizes this protein regardless of its activation status. The ‘overexpression’ of ADAM10 in MCL tumors _per se_ may be biologically important, since one previous report showed that overexpression of ADAM10 in colorectal cancer significantly correlates with a higher clinical stage 16. In this study, we demonstrated that 5 of 5 cases of MCL showing ADAM10 immunoreactivity also expressed the active/mature form of ADAM10. Taken together, it is likely that most MCL tumors overexpress ADAM10 as well as carry constitutive activation of this protein. Regarding our observation that ADAM10 can be localized to the nuclei of MCL cells, we would like to point out that a previous publication suggests that nuclear accumulation of ADAM10 in prostate cancer correlated with a higher Gleason score 40. Nevertheless, the biological and/or clinical significance of the nuclear localization of ADAM10 in MCL needs to be further studied.

Cyclin D1 overexpression due to t(11;14)(q13;q32), which brings the _cyclin D1(CCND1)_ gene under the influence of the enhancer of the immunoglobulin heavy chain (_IgH_) gene, is considered the hallmark for MCL 22. Cyclin D1 has been previously shown to be involved in the regulation of MCL cell proliferation 41. In the context of the role of ADAM10 in regulating cell cycle progression in MCL cells, we investigated if ADAM10 regulates the expression of cyclin D1 and other cell cycle-regulatory proteins including p21^waf1_. We observed that ADAM10 downregulation indeed resulted in a substantial downregulation of cyclin D1 and upregulation of p21^Waf1_, which correlated well with cell cycle arrest. Based on our observation that the downregulation of the cyclin D1 transcripts occurred within 24 hours after siRNA treatment, we believe that this change
in the cyclin D1 expression is directly due to the downregulation of ADAM10, rather than a consequence to the cell-cycle arrest. In view of the established link between the NFκB pathway and cyclin D1 as well as p21\(^{Waf1}\) \(^{42,43}\), it is possible that ADAM10 modulates the expression of cyclin D1 and p21\(^{Waf1}\) via activating the NFκB signaling pathway.

ADAM10 appears to enhance the growth-suppressing effect of bortezomib and MG132, two proteasome inhibitors. This observation is in keeping with the concept that proteasome inhibitor-induced apoptosis in MCL is at least partly mediated via a suppression of the NFκB signaling pathway \(^{37}\). Overall, our findings suggest that inhibition of ADAM10 may be a therapeutically useful strategy in treating MCL patients; specifically, inhibition of ADAM10 used in combination with proteasome inhibitors can enhance the overall tumor suppressive effects in MCL. Of note, pharmacologic inhibitors of ADAM10 are available, one of which is being tested in a clinical trial for patients with breast cancer \(^{10}\).

Different mechanisms have been shown to regulate the ADAM10 protein expression level; for instance, β-catenin has been shown to modulate the expression of ADAM10 in colon cancer \(^{44}\). Interestingly, β-catenin is known to be activated in a subset of MCL \(^{34}\). We tested if blockade of β-catenin using siRNA in MCL cell lines can result in a downregulation of ADAM10; no detectable change was observed (unpublished findings). Thus, the mechanism by which ADAM10 expression is modulated may well be cell-type specific. Regarding the activation of ADAM10, a number of enzymes are known to activate ADAM10, including proprotein convertase 7 and furin, which remove
the prodomain of ADAM10 \(^6\). The overexpression and/or aberrant activation of these enzymes in MCL may be involved in the activation of ADAM10. Further studies are needed to investigate the role of these proteins in MCL.

In conclusion, for the first time, our study describes that constitutive activation of ADAM10 is a consistent finding in MCL. We have provided evidence that ADAM10 contributes to the pathogenesis of MCL by activating the TNFα/NFκB signaling pathway. Lastly, we have provided evidence that inhibition of ADAM10 may be a useful approach to enhance the therapeutic effects of other agents (such as proteasome inhibitors) in treating MCL.
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AUTHORSHIP CONTRIBUTIONS

H.A. and P.G. designed research, performed experiments, analyzed data and wrote the manuscript. M.A. performed experiments. A.B. reviewed the manuscript and contributed to patients’ samples collection. R.L. designed research, analyzed data, wrote the manuscript and P.G. and R.L. coordinated the study.

DISCLOSURE OF CONFLICTS OF INTEREST:

The authors declare no conflict of interest.
REFERENCES:


FIGURE LEGENDS:

Figure 1: ADAM10 expression in MCL cell lines and patient samples

A) Western blots showed the expression of the precursor and active/mature form of ADAM10 in three MCL cell lines. While the precursor was highly expressed, the active/mature form was readily detectable only in Jeko-1 and Mino. A faint band at 72 kDa, representing the active/mature form of ADAM10, was also detectable in SP53. MCF-7 cells were used as the positive control. Peripheral blood mononuclear cells (PBMC) from a healthy individual (#1) showed abundant precursor ADAM10 protein but no detectable active/mature ADAM10. B) Western blots showed the presence of the precursor and active/mature form of ADAM10 in all MCL patients’ samples (both frozen #1-9 and leukemic #10-12); PBMC from two healthy individuals (#2 and #3) showed no detectable active/mature ADAM10. C) Immunohistochemistry showed no detectable signal of ADAM10 in the mantle zone of a reactive tonsil; the germinal centers showed faint staining, 100X (i). A case of MCL tumor showed no detectable ADAM10, 100X (ii). A MCL tumor showed a relatively high level of ADAM10 expression, 100X (iii). On high magnification, the ADAM10 immunostaining pattern was detectable in both of the nucleus and cytoplasm, 1000X (iv).

Figure 2: ADAM10 promoted cell growth in MCL

A) Western blots showed downregulation of the precursor and active/mature form of ADAM10 in Jeko-1 and Mino cells with the use of siRNA. B) ADAM10 knockdown induced significant inhibition of cell growth in Mino and Jeko-1 cells three days after transfection, as assessed by trypan blue exclusion assay (p=0.01 and 0.007,
respectively). Triplicate experiments were performed. C) ADAM10 knockdown induced significant inhibition of cell growth in Mino and Jeko-1 cells three days after transfection, as assessed by MTS assay (p<0.0001 and p=0.01, respectively). Triplicate experiments were performed. D) Addition of human recombinant ADAM10 (100 ng/ml) to SP53 and Mino cells induced a significant increase in their growth at 24 hours (p=0.008 and 0.01, respectively).

**Figure 3: ADAM10 induced cell cycle arrest**

Cell cycle analysis by flow cytometry using propidium iodide showed significant G₀/₁ cell cycle arrest in Mino cells following ADAM10 downregulation using siRNA. Of note, no appreciable increase in the fraction of cells in the subG₀/₁ was noted.

**Figure 4: ADAM10 regulated cyclin D1 expression in MCL**

A) Western blots showed downregulation of cyclin D1 and upregulation of p21Waf1 in Jeko-1 and Mino cells following ADAM10 downregulation using siRNA. B) Quantitative RT-PCR showed downregulation of the *cyclin D1* mRNA in Jeko-1 and Mino cells 24 hours after downregulation of ADAM10 using siRNA. C) Western blots showed upregulation of the nuclear level of cyclin D1 protein in SP53 cells 24 hours after the addition of human recombinant ADAM10 (100ng/ml); HDAC was used as the loading control for the nuclear extract.
Figure 5: ADAM10 activated the TNFα/ NFκB axis

A) Using an ELISA kit, we showed a significant downregulation of TNFα in Jeko-1 cells 48 hours after the transfection of ADAM10 siRNA, as compared to cells transfected with scrambled siRNA. B) Western blots showed a downregulation of phospho-NFκBp65 and total NFκBp65 after ADAM10 downregulation using siRNA, both in the total lysates harvested from Jeko-1 and Mino cells. C) Western blots showed a downregulation of total NFκBp65 and in nuclear extract from Jeko-1 and Mino cells. D) Using a NFκB reporter vector, dual luciferase assay, we showed a significant downregulation of the NFκB transcriptional activity in Mino cells after downregulation of ADAM10 using siRNA. E) Using NFκB reporter vector, dual luciferase assay, we showed a significant upregulation of the NFκB transcriptional activity after the addition of human recombinant ADAM10 (100 ng/ml) in Mino cells.

Figure 6: ADAM10 inhibition enhanced the growth suppressing effects of proteasome inhibitors bortezomib and MG132 in Jeko-1 cells

A) Jeko-1 cells transfected with either scrambled or ADAM10 siRNA were treated with MG132 (1 μM). At 24 hours after the MG132 treatment, trypan blue exclusion assay was performed and we found that ADAM10 siRNA enhanced the growth suppressing effect of MG132 in Jeko-1 cells (p <0.0001). B) Jeko-1 cells transfected with either scrambled or ADAM10 siRNA were treated with bortezomib (5 nM). At 24 hours after the bortezomib treatment, trypan blue exclusion assay was performed and we found that ADAM10 siRNA enhanced the growth suppressing effect of bortezomib in Jeko-1 cells (p <0.0001, ANOVA).
Fig. 1A

MCL cell lines

<table>
<thead>
<tr>
<th>Jeko-1</th>
<th>Mino</th>
<th>SP53</th>
<th>MCF-7</th>
<th>PBMC #1</th>
</tr>
</thead>
</table>

ADAM10

- Precursor form 98 kDa
- Mature form 72 kDa

β-actin

- 43 kDa
Fig. 2A

Jeko-1

- Scrambled siRNA
- siRNA ADAM10

ADAM10: Precursor form 98 kDa, Mature form 72 kDa

β-actin: 43 kDa

Mino

- Scrambled siRNA
- siRNA ADAM10

ADAM10: Precursor form 98 kDa, Mature form 72 kDa

β-actin: 43 kDa
Fig. 2B

Mino

% of cell count relative to control

Scrambled siRNA  ADAM10 siRNA

p=0.01

Jeko-1

% of cell count relative to control

Scrambled siRNA  ADAM10 siRNA

p=0.007
Fig. 2C

Scrambled siRNA  |  ADAM10 siRNA
---|---
Mino

\[ p<0.0001 \]

Jeko-1

\[ p=0.01 \]
Fig. 2D

SP53

Mino

% of cell count relative to control

p=0.008

p=0.01

Control
Recombinant ADAM10

Control
Recombinant ADAM10
Fig. 3

<table>
<thead>
<tr>
<th></th>
<th>% G1</th>
<th>%S</th>
<th>%G2</th>
<th>Total</th>
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<tbody>
<tr>
<td>Control</td>
<td>34.43 ± 2.3</td>
<td>54.44 ± 4.2</td>
<td>11.13 ± 6.4</td>
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</tr>
<tr>
<td>siADAM10</td>
<td>53.6 ± 4.4</td>
<td>35.69 ± 1.1</td>
<td>10.71 ± 5.5</td>
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</table>
**Fig. 4A**

<table>
<thead>
<tr>
<th>siRNA ADAM 10</th>
<th>Jeko-1</th>
<th>Mino</th>
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<tbody>
<tr>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **Cyclin D1**
  - Jeko-1: Image
  - Mino: Image
  - 36 kDa

- **β-actin**
  - Jeko-1: Image
  - Mino: Image
  - 43 kDa

- **p21**
  - Jeko-1: Image
  - Mino: Image
  - 21 kDa

- **β-actin**
  - Jeko-1: Image
  - Mino: Image
  - 43 kDa
Fig. 4B

**Cyclin D1**

For Jeko-1:
- ADAM10 siRNA: ~70-fold increase
- Scrambled siRNA: ~90-fold increase

For Mino:
- ADAM10 siRNA: ~100-fold increase
- Scrambled siRNA: ~50-fold increase

**ADAM10**

For Jeko-1:
- ADAM10 siRNA: ~120-fold increase
- Scrambled siRNA: ~40-fold increase

For Mino:
- ADAM10 siRNA: ~120-fold increase
- Scrambled siRNA: ~20-fold increase
Fig. 4C

Recombinant ADAM10 100ng/ml

<table>
<thead>
<tr>
<th></th>
<th>Nucleus</th>
<th>Cytoplasm</th>
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<tbody>
<tr>
<td>0 24h</td>
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<tr>
<td>Cyclin D1</td>
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<tr>
<td>HDAC</td>
<td><img src="#" alt="Image" /></td>
<td><img src="#" alt="Image" /></td>
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<tr>
<td>α-tubulin</td>
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</table>

SP53

- 36 kDa
- 65 kDa
- 55 kDa
Fig. 5A

Jeko-1

<table>
<thead>
<tr>
<th></th>
<th>Mean (pg/ml) ± Standard deviation</th>
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<tr>
<td>Scrambled siRNA</td>
<td>4.56 ± 0.3</td>
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<tr>
<td>siRNA ADAM10</td>
<td>2.45 ± 0.3</td>
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</table>

p=0.001
Fig. 5B

<table>
<thead>
<tr>
<th>siRNA ADAM 10</th>
<th>Mino</th>
<th>Jeko-1</th>
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<tr>
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<tr>
<td>Phospho-NFkBp65</td>
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<td><img src="image2" alt="Image" /></td>
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<tr>
<td>NFkBp65</td>
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<tr>
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<td>β-actin</td>
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<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>43 kDa</td>
<td></td>
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</tbody>
</table>
Fig. 5C

**Mino**

<table>
<thead>
<tr>
<th>siRNA ADAM10</th>
<th>Nucleus</th>
<th>Cytoplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

**Jeko-1**

<table>
<thead>
<tr>
<th>siRNA ADAM10</th>
<th>Nucleus</th>
<th>Cytoplasm</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

NFκBp65

HDAC
Fig. 5D

Mino

Fold change in luciferase activity

Scrambled siRNA
ADAM10 siRNA

p = 0.003

Fig. 5E

Mino

Fold change in luciferase activity

Control
Recombinant ADAM10

p = 0.0004
Fig. 6A

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% of cell count relative to control

- Scrambled siRNA
- ADAM10 siRNA
- Scrambled siRNA + Bortezomib
- ADAM10 siRNA + Bortezomib

p < 0.0001

Jeko-1
Constitutive activation of metalloproteinase ADAM10 in mantle cell lymphoma promotes cell growth and activates the TNF α/NFκB pathway

Hanan Armanious, Pascal Gelebart, Mona Anand, Andrew Belch and Raymond Lai