Prevention of Mantle Lymphoma Tumor Establishment by Routing Transferrin Receptor toward Lysosomal Compartments

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Abstract

Mantle cell lymphoma (MCL) is one of the most frequent of the newly recognized non-Hodgkin's lymphomas. The major problem of MCL therapy is the occurrence of relapse and subsequent resistance to chemotherapy and immunotherapy in virtually all cases. Here, we show that one injection of anti-human transferrin receptor (TfR) monoclonal antibody A24 totally prevented xenografted MCL tumor establishment in nude mice. It also delayed and inhibited tumor progression of established tumors, prolonging mice survival. In vitro, A24 induced up to 85% reduction of MCL cell proliferation (IC50 = 3.75 nmol/L) independently of antibody aggregation, complement-dependent or antibody-dependent cell-mediated cytotoxicity. A24 induced MCL cell apoptosis through caspase-3 and caspase-9 activation, either alone or synergistically with chemotherapeutic agents. A24 induced TfR endocytosis via the clathrin adaptor protein-2 complex pathway followed by transport to lysosomal compartments. Therefore, A24-based therapies alone or in association with classic chemotherapies could provide a new alternative strategy against MCL, particularly in relapsing cases. [Cancer Res 2007;67(3):1145–54]

Introduction

Mantle cell lymphoma (MCL) is a distinctive non-Hodgkin's lymphoma subtype (1) that is well recognized by both European-American classification of lymphoid neoplasms (REAL; ref. 1) and WHO (2). Despite its morphologic heterogeneity, MCL is defined as a lymphoma of small lymphocytes with a characteristic phenotype (CD5+, CD23-, CD10-, IgM+, and IgD+) associated with a juxtaposition of the BCL1 gene on chromosome 11 with the immunoglobulin heavy-chain gene on chromosome 14, the so-called t(11,14) (q23,q32) translocation. This juxtaposition mediates the overexpression of the cyclin D1 gene (3) that contributes to the establishment and development of this malignancy. MCL is an aggressive disease associated with poor prognosis and responsiveness to conventional chemotherapy with a 3- to 4-year median survival (4). First-line intensive high-dose chemotherapy, including high-dose cytosine arabinoside (ara-C) regimen, may induce complete remission in a significant number of cases. High-dose chemotherapy has improved significantly survival and event-free survival. However, the probability of cure remains low since relapse occurred in virtually all cases (5, 6). Novel therapeutic approaches, including monoclonal antibody (mAb)–based therapy (such as anti-CD20, rituximab), significantly increase response rates, but the mean survival is not drastically improved (7–9). Recent trials with the proteasome inhibitor PS341 (bortezomib) have shown a good response rate in relapsing MCL patients (10). However, the duration of response is not yet known, and relapses occur on therapy. Therefore, new therapeutic agents are needed to achieve efficient treatment of MCL, particularly to prevent relapse, which is the major challenge of this still incurable disease.

Transferrin (Tf) is the major serum iron transporter. Iron plays a central role in a large number of essential cellular functions, such as oxygen transport, mitochondrial energy metabolism, and electron transport. Rapidly growing tumor cells, but not their normal counterparts, highly express Tf receptors (TfR) and require transferrin for cell growth and survival. TfR promotes iron uptake through endocytosis of Fe-loaded holotransferrin molecules and are constantly recycled to the cell surface (11). Fe-Tf-induced TfR endocytosis is dependent on clathrin-coated pits. In these membrane microdomains, several accessory proteins have been implicated in Fe-Tf-dependent receptor endocytosis. Among clathrin-associated proteins, the adaptor protein-2 (AP-2) complex is, at least in part, responsible for TfR sorting to the endocytic compartment (12, 13). In this regard, AP-2 depletion in HeLa cells induces TfR up-regulation due to endocytosis impairment (14).

We recently reported the ability of a new anti-TfR mAb (A24) to specifically target highly proliferative cells (15). Although non-blastic MCL is considered to some extent as an indolent lymphoma, cells from MCL patients are characterized by their profile of proliferation gene expression (16). Thus, we hypothesized that MCL may highly express TfR and, as such, might be a good candidate to be targeted by A24 antibody in vitro and in vivo. Herein, we show that TfR is highly expressed on MCL cells, and that A24 totally prevented tumor establishment and decreased the growth rate of pre-established tumors in athymic nude mice. In vitro, A24 blocked proliferation of these malignant cells by inducing cell apoptosis through caspase-3 and caspase-9 activation. A24 induced an AP-2–dependent TfR endocytosis, leading to its translocation to lysosomal compartments. These observations open new avenues in the treatment of this aggressive disease, particularly in its relapsing stage.
Materials and Methods

Cells, antibodies, and chemical agents. Granta-519, NCEB-1, REC-1, JVM-2 (17), UPN1, and UPN2 (18) MCL cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mmol/L glutamine, and 50 μg/mL penicillin/streptomycin, at 37 °C in a humidified atmosphere containing 5% CO2. Anti-CD20-FITC, anti-TR-phycocerythrin (PE), irrelevant mouse IgG-FITC, and mouse IgG-PE isotype controls were all from Immunotech (Marseille, France). Chemotherapeutic agents Adriablastin (Pharmacia, Brussels, Belgium), ara-C (Pharmacia, Belgium), etoposide (VP-16; Sigma, Lyon, France) were used at 0.1 to 1,000 ng/mL. Blocking antibodies rituximab (anti-CD20; Roche Diagnostic, Meylan, France) and A24 anti-human TfR (15) were used.

Patient biopsies and immunostaining of sections. Lymph node biopsies were obtained from the Anatomopathology Department at the Necker Hospital after agreement of the Necker Hospital ethical committee and after patients' written informed consent. Tumor cells were isolated from ascite with a peritoneal involvement by MCL cells (CD5+, CD23+, FMC7+, cyclin D1+, and IgH-bcl1; data not shown). Biopsies were from diffuse large B cell lymphoma (DLBCL), small lymphocytic lymphoma (SLL), follicular lymphoma (FL), and MCL. Patients' cells were classified according to histologic morphologic and immunophenotypic (CD5+, CD20+, and cyclin D1+) criteria. Cryostat sections from patients' biopsies were blocked in TBS/2% nonfat milk, stained with 10 μg/mL A24 or anti-Ki-67 in PBS/0.5% bovine serum albumin, and incubated in a humidified chamber for 30 min at room temperature. The primary antibody was visualized using an additional secondary antibody. The DAKO LSAB system was used for the staining procedure of aceton-exfixed cryostat sections according to the manufacturer's instructions.

Gene knockdown. A strategy using pTRIP.CMV.GFP lentivirus vector for short hairpin RNA (shRNA) delivery was chosen. The lentivirous vector was kindly provided by Dr. Pierre Charneau (19). For the construction of pTRIP-AP-1-γ adaptin or pTRIP-AP-2-μ2, a DNA fragment containing H1 promoter and AP-1 or AP-2 shRNA sequence was generated by SpeI/SalI double digestion of pSUPER-AP-1-γ adaptin or AP-2-μ2 plasmids (20) and was subcloned into pTRIP.CMV.GFP at the HaelI/SalI cloning site, which is located within the 3′ long terminal repeat of the vector.

For virus production, the 293T human kidney cells (2×10⁶) were seeded on 10-cm plates and transfected the following day with 15 μg of packaging plasmid pCMVΔR8.71 (21), 20 μg of the various pTRIP-CMV-GFP plasmid constructs (AP-1-γ adaptin, AP-2-μ2, or control), and 5 μg of VSV-G-coding plasmid pHCMVG (ref. 22; kindly provided by Prof. Didier Trono), using calcium phosphate DNA precipitation method. Eight hours after transfection, the medium was replaced, and after 24 h, cells were placed at 32 °C for 3 days. For virus infection, the medium was removed, and the cells were placed at 37 °C to increase viral titer. Forty-eight hours after transfection, the 0.45-μm filtered supernatant was collected and conserved at −80 °C before use for infection.

The DG-75 human B cell line (23), kindly provided by Prof. Martin Rowe, was then infected with the various lentivirus. Cells were cultured in RPMI 1640 containing 10% FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies, Cergy Pontoise, France). Five hundred thousand cells were resuspended in 200 μL culture medium and used for each infection. The viral supernatant was added in triplicate at 2.5×10⁴ per well in 96-well tissue culture plates (Falcon, Oxnard, CA). Proliferation was measured over 18 h, using pulses of 1 μCi/well (0.037 MBq/well) [3H]thymidine (Amersham, Saclay, France). Cells were harvested with a 96-well Harvester (Pharmacia) and collected on filters (Pharmacia), and incorporation of [3H]thymidine was measured with a β-plate microscintillation counter (LKB, Pharmacia, France).

Cell cycle analysis. Cells were cultured for 24, 48, or 72 h in the presence of different chemotherapeutic agents, each used at its IC₅₀ concentration. Cells were then harvested and centrifuged, resuspended in a propidium iodide buffer (0.1% of NaCl, 0.1% Triton X-100, 50 μg/mL propidium iodide), and incubated 15 min at 37 °C. The reaction was stopped by the addition of cold PBS. Cell cycles were then analyzed using a flow cytometer (FACSCalibur, Becton Dickinson, Le pont de Claix, France).

Apoptosis assay. Phosphatidylserine externalization was evaluated by Annexin V (Becton Dickinson) binding as previously described (15). Apoptotic cells were visualized by Annexin V/propidium iodide double staining. For activated caspase-3 detection, cells were cultured in the presence of A24, ara-C, or medium. Cells were then harvested, washed twice in PBS, and fixed in 4% paraformaldehyde for 5 min at 4 °C. After fixation, cells were stained with anti-caspase-3 antibody or control isotype during 30 min in the presence of 0.1% saponin, 0.4% bovine serum albumin in PBS. Caspase-3 staining was revealed using a donkey anti-mouse IgG-PE.

Western blot analysis. Treated and untreated MCL cells were lysed using Laemmli sample buffer [62 mmol/L Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% (v/v) β-mercaptoethanol] and boiled for 3 min. Protein content was measured using the Bio-Rad Protein assay, and 30 μg per lane were loaded on a 12% SDS-PAGE gel. Proteins were transferred onto polyvinylidene difluoride membrane (Millipore, Saint-Quentin en Yvelines, Belgium) and were detected with polyclonal rabbit antibody against cleaved caspase-3, cleaved caspase-9, p27kip, cyclin D1, γ-adaptin, μ2, tubulin, or actin (Tebu, Le Perray-en-Yvelines, France) followed by a secondary antibody-horseradish peroxidase conjugate (Pierce, Brevières, France) and visualized by chemiluminescence with the ECL kit (Pierce).

TRF internalization. Both EBV− and EBV+ MCL cells were incubated at 4 °C with A24 or rituximab for 30 min, washed, and plated at 37 °C in culture medium for various time lengths (30 min to 72 h). Cells were then harvested and stained with a mAb against TRF or against CD20 or with control isotype (all from Becton Dickinson) and analyzed by flow cytometry.

Confocal microscopy. MCL cells were incubated with A24 and plated in culture medium at 37 °C for 15 min to 1 h. Lysotracker experiments were done according to the manufacturer's instructions (Molecular Probes, Cergy Pontoise, France). Controls for cell surface labeling in the absence of receptor internalization were done at 4 °C. Cells were harvested, washed, and plated on poly-L-lysine (Sigma, St. Louis, MO)—coated slides for 1 h at 4 °C. Cells were fixed in 4% paraformaldehyde, quenched with 0.1 mol/L glycine, and permeabilized with 0.5% saponin. To detect A24, slides were incubated with a polyclonal goat anti-mouse IgG conjugated to CY5 (Jackson ImmunoResearch, Villepinte, France). Lysosomal compartments were identified using monoclonal anti-LAMP-1 (CD107a-FITC) and anti-LAMP-2 (CD107b-PE) antibodies (Becton Dickinson). Mounted slides were examined with a confocal laser microscope (LSM 510, Carl Zeiss, Jena, Germany).

Human tumor xenografts. For prevention of tumor establishment, 5×10⁶ UPN1 cells mixed with Matrigel (1:1, v/v) were injected as s.c. xenografts in 30 female athymic (NCR nu/nu, Taconic, Germantown, NY) nude mice sublethally irradiated (5 Gy). Mice were then i.v. injected with A24 (40 mg/kg) or PBS as a vehicle control. For treatment of established tumors, 19 mice bearing tumors, reaching a diameter of about 5 mm, were injected i.v. with A24 (40 mg/kg) or PBS. Tumor growth was measured twice a week. Tumor volume was calculated using the following equation: V = L × S²/6, where L is the longer, and S is the shorter, of the two dimensions. Tumor volumes were then expressed as a percentage of the volume at the start of treatment. The effect of the drug was determined by the growth delay as previously described (24).

Results

TRF is overexpressed in MCL cells infiltrating human lymph nodes and in MCL cell lines. TRF expression is enhanced in highly proliferative tumor cells (25). It has been previously shown that TRF is expressed in non-Hodgkin's lymphomas (26). However, there is no extensive data about in situ TRF expression on different histologic subtypes of B cell non-Hodgkin's lymphomas, including MCL. Although most MCL are closely related to low-grade lymphomas, they are characterized by overexpression of the cyclin D1 gene. Therefore, TRF expression was compared between MCL and other B cell non-Hodgkin's lymphomas. Two main groups of
indolent lymphomas (i.e., SLL and follicular lymphoma low-grade lymphoma) and the main subtype of aggressive lymphomas (i.e., DLBCL), were studied. Among non-Hodgkin's lymphomas, TIR was highly expressed in all DLBCL (five of five biopsies; Fig. 1A, a). By contrast, SLL (five biopsies) and follicular lymphoma (five biopsies) poorly expressed TIR (Fig. 1A, b; data not shown). Interestingly, we found TIR expression in five of five biopsies derived from MCL patients (Fig. 1A, c). However, the number of TIR-positive cells and the intensity of TIR labeling were variable among MCL biopsies. To examine if these differences in TIR expression were correlated to tumor proliferation status, we compared TIR expression with that of the Ki-67 proliferation marker. Figure 1B shows immunohistochemical analysis of high (a and d), medium (b and e), and low (c and f) mitotic activity. The levels of TIR and Ki-67 expression were correlated because TIR expression was high in Ki-67\(^{\text{high}}\) biopsies and weak when low numbers of Ki-67-positive cells were detected. As reported earlier (27), TIR expression was also observed in reactive activated T cells.

Figure 1. TIR expression on MCL.
A, immunohistochemical analysis of TIR expression with A24 in DLBCL (a), SLL (b), and MCL B-cell (c). Magnification, ×400. 
B, TIR is correlated with Ki-67 expression in MCL. Immunohistochemical analysis of TIR and Ki-67 expression on lymph nodes biopsies from MCL patients. Ki-67 high (d), medium (e), and low (f) correlates with TIR staining (a-c).
C, TIR is highly expressed in MCL patient cells (a) but not on healthy individuals (b). TIR and CD5 expression on MCL cells (CD5\(^{+}\), CD23\(^{-}\), FMC7\(^{-}\), cyclin D1\(^{-}\), and IgH-bcl1\(^{-}\)) infiltrating ascite. D and E, enhanced TIR and CD20 expression in established-MCL lines. MCL cells (NCEB-1 (a), JVM-2 (b), UPN2 (c), UPN1 (d), REC-1 (e), and Granta-519 (f)) were stained with monoclonal antibodies (closed histograms) against TIR (D) or CD20 (E) and with control isotype (open histograms). Numbers indicate the median fluorescence intensity values.
present in the lymph node (data not shown). We next assessed the level of TfR expression in MCL cells from patients. Ascite infiltrated by MCL (CD5+) cells were highly positives (89%) for TfR compared with control cells (Fig. 1C).

Figure 2. In vivo action of A24 in nude mice bearing xenografted tumors. A, A24 treatment prevents tumor establishment. Kaplan-Meier survival curves of sublethally irradiated nude mice s.c. injected with UPN1 cells and treated with A24 (40 mg/kg) or vehicle (PBS). B, A24 treatment delayed tumor growth. Growth curves from pre-established UPN1 tumors (~5 mm) in mice treated with A24 or vehicle (PBS). Points, mean; bars, SE. C, A24 enhances the survival of mice bearing MCL tumors. Kaplan-Meier survival curves of mice bearing pre-established UPN1 tumors treated with A24 or vehicle (PBS).

Figure 3. A24 blocks MCL cell proliferation and synergizes with anti-MCL chemotherapeutic agents. A, A24 inhibits MCL tumor cell proliferation. Both EBV+ and EBV− MCL cell lines were cultured for 72 h in the presence of rituximab (0.625–10 μg/mL) or A24 (0.625–10 μg/mL). Cells were then pulsed for 16 h with [3H]thymidine and harvested to measure radioactive incorporation. B and C, A24 can act in synergy with MCL chemotherapies. EBV+ and EBV− MCL were incubated with or without A24 (0.625 μg/mL) in the presence of chemotherapeutic agents, such as ara-C (1 ng/mL), VP-16 (100 ng/mL), and Adriablastin (4 ng/mL) for 72 h to determine the potential synergistic effect of these agents with A24. Cell proliferation was then evaluated by [3H]thymidine incorporation. Columns, mean of one representative experiment out of three separate experiments; bars, SE.
proliferative characteristics of MCL in comparison with other indolent lymphomas.

EBV transformation is widely used to generate MCL cell lines; thus, MCL tumor cell lines derived from patients are generally EBV positive. Therefore, as a first step, to avoid confounding factors due to EBV infections, we screened MCL cell lines to diagnose EBV infection (28). Four of five MCL cell lines (REC-1, Granta-519, JVM-2, and NCEO-1) were found positive for EBV, and only one MCL cell line (UPN1) was EBV negative (data not shown). Therefore, Granta-519 and UPN1 were used in the subsequent experiments to analyze both EBV-infected and non-infected MCL cells. Phenotypic analysis showed that Tfr and CD20 are expressed on both EBV+ and EBV− MCL cells (Fig. 1D and E). Taken together with immunohistochemistry data from MCL patients, these results reveal the high level of Tfr expression in MCL cells in vivo and in vitro.

A24 prevents tumor establishment and development in athymic nude mouse model. We established an in vivo model to evaluate A24 effects on MCL tumor development. The MCL cell line UPN1 was injected s.c. in nude mice followed by one single i.v. injection of A24 (40 mg/kg) or vehicle as a control. Eight days after tumor cell injection, all mice in the control group had developed tumors (20 of 20) and died 2 weeks later. Remarkably, none of the A24-treated mice (10 of 10) developed tumors; consequently, none of them died (Fig. 2A).

We then tested the effect of A24 on the growth rate of MCL (UPN1) tumors. Mice bearing established tumors were treated with only one dose of A24 (40 mg/kg; 10 mice) or excipient alone (9 mice). As shown in Fig. 2B, injection of a single dose of A24 in mice bearing pre-established tumors significantly delayed tumor growth compared with controls after a lag time of 3 days. Additionally, it significantly slowed down tumor growth because tumors in A24-treated mice grew with roughly half the rate of the tumors in control mice. A24 single injection also increased mice survival (Fig. 2C). Twenty percent of the A24-treated mice still survived 24 days after injection of the antibody, whereas all control mice had died by day 17. Collectively, these results underscore the potent anti-MCL activity of A24 and open the possibility that A24-derived immunotherapy could be efficient in preventing relapse and could prevent primary tumor growth and disease progression.

A24 and chemotherapeutic agents affect MCL cell proliferation. Because A24 had proven in vivo efficient antitumor properties, a series of in vitro experiments were carried out to study in greater detail its mechanism of action on MCL cells. We compared the effect of an antibody directed against the B-cell marker CD20 (rituximab), validated in human therapy, with that of A24 on the proliferation of MCL cell lines. Granta-519 (EBV+) and UPN1 (EBV−) cells were cultured for 72 h in the presence of A24 or rituximab at various concentrations (0.625–10 μg/mL), and cell proliferation was evaluated by [3H]thymidine incorporation. Both MCL cell lines were sensitive to A24, and 85% inhibition of cell proliferation was reached at a low concentration of antibody (1.25 μg/mL; Fig. 3A). By contrast, rituximab that relies mostly on complement-dependent and/or antibody-directed cell cytotoxicity

Figure 4. A24 induces apoptosis of MCL cells. A and B, MCL chemotherapeutical agents (ara-C and VP-16), but not A24, induce cell cycle arrest. MCL cells were cultured in the presence of VP-16 (100 ng/mL) and ara-C (1 ng/mL), rituximab (10 μg/mL), or A24 (10 μg/mL) for 24 h. Cell cycle was assessed by flow cytometry using propidium iodide after Triton X-100 permeabilization to determine G0-G1, S, and G2-M phases. C, A24 does not alter expression of cell cycle cyclin D1 or p27kip proteins. Protein extracts obtained from UPN1 MCL cell line cultured 72 h in the absence or the presence of A24 were resolved by SDS-PAGE and blotted using anti-cyclin D1 and anti-p27kip antibodies. Antitubulin antibodies were used as a control for sample equal loading. D–F, A24 induces apoptosis of MCL cells. UPN1 cells were cultured in the absence or in the presence of 10 μg/mL A24. Apoptosis was evaluated by Annexin V and propidium iodide staining. Numbers indicate the percentage of apoptotic cells. E, MCL cells were cultured in the presence of medium, ara-C (4 ng/mL), or A24 (10 μg/mL). Apoptosis was examined after 24 h by flow cytometry using a mAb against the cleaved form of caspase-3 revealed with an anti-mouse-PE (closed histograms) and compared with corresponding control isotype-PE (open histograms). F, MCL cells were cultured in the presence of medium or A24 (10 μg/mL). Apoptosis was examined by immunoblotting using anti-caspase-9 antibody. Actin immunoblotting was used to control for equal protein loading.
(ADCC; ref. 29) induced only a minor effect on cell proliferation (up to 30% inhibition at 10 μg/mL).

Previous studies showed that rituximab improves the prognosis of MCL when combined with chemotherapy (30). We therefore examined the combined action of A24 with conventional anti-MCL chemotherapeutic agents. Pharmacologic concentrations of doxorubicin (Adriblastin), ara-C (Aracytine), and VP-16 (etoposide) were tested in vitro in combination with the IC50 concentration of A24 in both EBV+ and EBV− cell lines. Whereas A24 promoted an inhibition of proliferation of both MCL cell lines at least comparable with VP-16 and ara-C, Adriblastin showed only a marginal effect on EBV− and had no effect on EBV+ cells. Interestingly, the combined effect of A24 with VP-16 or with ara-C was significantly more pronounced than that of any of these agents alone. This difference was more markedly observed in EBV+ cells (Fig. 3B and C). Thus, A24 showed an inhibiting effect on MCL cell proliferation that was both comparable with and synergistic with conventional chemotherapeutic agents.

**A24 induces apoptosis but not cell cycle arrest of MCL cells.** Overexpression of cyclin D1 is the hallmark of MCL, and translocation of this gene is correlated with the molecular proliferative signature of the disease. It is currently admitted that overexpression of cyclin D1 results in the sequestration of the p27kip cell cycle inhibitor, thus enhancing proliferation of these tumor cells (31). Given these particular alterations in cell cycle, several studies have been concentrated on the use of cell cycle inhibitors as therapeutic agents of MCL. We therefore compared the cell cycle status of MCL cells treated by ara-C, VP-16, rituximab, and A24 (Fig. 4A and B). As it is well established, ara-C and VP-16 block MCL cells in S and G2-M phases, respectively. A24, however, did not block the cell cycle at any particular stage. Accordingly, cyclin D1 expression was not down-regulated in MCL cells treated with A24 (Fig. 4C).

The absence of the effect of A24 on the cell cycle prompted us to investigate its proapoptotic effect. Apoptosis of MCL cells was evidenced by analysis of phosphatidylserine externalization and
caspase activation. A24 induced cell apoptosis observed by phosphatidylserine externalization (50% Annexin V+ cells versus 15% Annexin V- cells; Fig 4D). Both ara-C and A24 induced cleavage of caspase-3 in 50% and 60% of cells, respectively, which was highly significant compared with control cells (12%; Fig 4E). Additionally, A24 also induced caspase-9 cleavage (Fig. 4F). Thus, A24 induced MCL cell apoptosis without affecting the cell cycle at any particular phase.

A24 induces TfR endocytosis to the lysosomal compartment dependent of AP-2 complex. TfR is the paradigm of receptor-mediated endocytosis. Under physiologic conditions, TfR endocytosis is induced by Fe-loaded (holo)-transferrin (Fe-Tf). However, it has been shown previously that TfR capping by anti-TfR antibodies has been shown to mainly participate in budding of clathrin-coated vesicles and recycling endosomes (pH 6.4). After iron dissociation, apotransferrin and TfR recycle back to the cell surface stained with anti-TfR and anti-CD20 antibodies recognizing epitopes that do not overlap with A24 induced receptor down-regulation are AP-2 and clathrin dependent. For this purpose, we used shRNA constructs in recombinant lentivirus vector to knock down the μ2 subunit of AP-2 heterotrimeric adaptor complexes, and as a control, we knocked down the γ-adaptin subunit of AP-1 adaptor complexes that were previously shown to mainly participate in budding of clathrin-coated vesicles from the trans-Golgi network. These shRNA sequences specifically target μ2 and γ-adaptin genes to induce protein knockdown (20).

A24 endocytosed TfR and lysosomotropic probe (LysoTracker). UPN1 cells were incubated at 4°C with A24 and plated under culture conditions at 37°C for 60 min. Fifteen minutes before incubation, LysoTracker (green) was added to decorate acidic compartments. After labeling, A24/TfR complexes visualized by a goat anti-mouse-Cy3 (red). Original magnification, ×400. E, decreased expression of μ2 and γ-adaptin in lentivirus vector transduced cells. Western blot analysis of μ2 and γ-adaptin expression in DG-75 cells infected with lentivirus coding for μ2 or γ-adaptin shRNA or with mock lentivirus (encoding for GFP only). Antitubulin immunoblotting was used to control for equal protein loading. F, AP-2 depletion abrogates TfR internalization. Lentivirus coding shRNA for AP-1 or AP-2 target sequences or mock lentivirus were used to infect DG-75 cells. Transduced cells were then subjected to A24-induced TfR internalization, and TfR membrane expression was assessed by surface labeling.

Figure 5 Continued. D, colocalization of A24-endocyotised TfR and lysosomotropic probe (LysoTracker). UPN1 cells were incubated at 4°C with A24 and plated under culture conditions at 37°C for 60 min. Fifteen minutes before incubation, LysoTracker (green) was added to decorate acidic compartments. After incubation, A24/TfR complexes visualized by a goat anti-mouse-Cy3 (red). Original magnification, ×400. E, decreased expression of μ2 and γ-adaptin in lentivirus vector transduced cells. Western blot analysis of μ2 and γ-adaptin expression in DG-75 cells infected with lentivirus coding for μ2 or γ-adaptin shRNA or with mock lentivirus (encoding for GFP only). Antitubulin immunoblotting was used to control for equal protein loading. F, AP-2 depletion abrogates TfR internalization. Lentivirus coding shRNA for AP-1 or AP-2 target sequences or mock lentivirus were used to infect DG-75 cells. Transduced cells were then subjected to A24-induced TfR internalization, and TfR membrane expression was assessed by surface labeling.
The extent of specific depletions was evaluated by immunoblot analysis that compared the levels of these proteins with that in cells transduced with mock [encoding for green fluorescent protein (GFP) only] lentivirus vector as a control. Infection of cells with AP-1 and AP-2 shRNA-lentivirus vectors significantly reduced expression of the targeted proteins (Fig. 5E). Cells knocked down for either adaptor complexes were then incubated with A24 to determine if TfR endocytosis mediated by this antibody was dependent on AP-2. AP-2 depletion resulted in a dramatic reduction of A24-induced TfR internalization (Fig. 5F). By contrast, AP-1 depletion did not significantly alter TfR modulation. Therefore, the initial step of A24-induced TfR endocytosis followed the same initial intracellular route as those used by Fe-Tf/TfR complexes.

Discussion

MCL accounts for 6% to 7% of non-Hodgkin’s lymphomas and is characterized by a proliferation gene expression phenotype that involves cyclin D1 overexpression (4, 34). MCL patients have a high degree of primary and secondary treatment resistance, and this disease remains the lymphoma subtype with the poorest long-term outcome and with the highest fraction of death from lymphoma (35, 36).

In spite of recent progress in MCL treatment, the disease prognosis remains poor mainly due to the intrinsic resistance of malignant cells to conventional chemotherapy (37–39), and virtually, all patients, even those who exhibit complete response, will relapse. Because CD20 antigen is expressed in the majority of non-Hodgkin’s lymphomas, including MCL, immunotherapy with anti-CD20 antibodies (rituximab) have been used (40, 41). However, in contrast with the broad expression of CD20, rituximab alone has been successful to induce partial or complete responses in only 20% to 40% of MCL patients (42, 43). Although rituximab immunotherapy in association with conventional chemotherapy increased response rate in MCL, its beneficial effect on overall survival remains to be shown (5, 30). More importantly, rituximab-relapsed MCL patients lose CD20 expression on tumor cells, presenting subsequently both chemotherapeutic resistance and rituximab unresponsiveness. Thus, discovering new therapeutic approaches to increase significantly response rate and to prevent relapse remains the major challenge in MCL (44).

Given the importance of TfR in iron uptake, impairing this function may promote cell death (45, 46). TfR is necessary for cell proliferation and is highly expressed on rapidly growing tumor cells, in particular in solid tumors (47), but not on their normal counterparts, supporting TfR as an antitumoral target. The data presented herein suggest that A24-based therapy might be useful not only in blocking MCL tumor growth but also in a preventive setting to tumor relapses. Our in vivo experiments show that A24 can act on pre-established tumors because A24 was efficient in decreasing tumor growth rate and in extending mice survival. Other studies in animal models have been done using other anti-TfR blocking antibodies such as mAb 42.6. Although this mAb is effective in vitro on cultured tumor cells, it did not induce significant effects on solid tumors (48). However, in humans, phase Ia trial with mAb 42.6 was well tolerated, highlighting the safety of anti-TfR immunotherapy, the major side effects of this treatment being an increased serum iron and Tf saturation (49). Yet, no partial or complete remission of tumor growth was observed. By contrast, here, we show that A24 is able to target tumor cells in vivo in a solid tumor context.

Based on the evidence provided herein, immunotherapeutic strategies derived from the anti-TfR mAb A24 are expected to have a strong potential in preventing relapse in MCL patients. Indeed, in vivo administration of A24 completely prevented tumor establishment in all the mice tested (10 of 10). This was achieved with one single injection of the antibody, whereas all mice that had been treated with the vehicle alone died within 2 weeks as a consequence of tumor development (20 of 20). Our immunohistochemical analysis revealed that TfR and Ki-67 expression were correlated. Interestingly, Ki-67 expression was previously associated to a shorter survival of patients (50). In addition, it was recently shown that the TfR gene is up-regulated in MCL relapses (51). Therefore, it is expected that A24-based immunotherapy may be even more efficient to target chemotherapeutic resistant clones in relapsing patients. Thus, based on our murine in vivo preventive model A24-derived therapies might be efficient to prevent relapse in the setting of minimal residual tumoral cells such as that observed after first-line treatments of the disease.

We have previously established that A24 competes with transferrin for receptor binding with K equal to 2.7 nmol/L. A24 cytotoxicity was shown to be restricted to malignant cells because a high density of TfR expression is required for its efficient binding, whereas in nonmalignant cells expressing low receptor levels, binding of transferrin over A24 is favored (15). Thus, A24-targeted cells are largely ineffective to endocytose Fe-Tf and consequently enter apoptosis similarly to that induced by iron-chelating agents (15).

In an attempt to improve outcome of MCL, various drug combinations, including alkylating agents, anthracyclines, and purine analogues, have been explored in MCL patients. They all failed to substantially improve the long-term survival (52). Resistance to alkylating agents and to anthracycline could be due to π-class glutathione S-transferase, whose gene located in 11q13 could be coamplified along with the cyclin D1 gene (53). Here, the ability of A24 to inhibit MCL cell proliferation was comparable with that of ara-C or of VP-16, and their combined action was even stronger. In contrast to chemotherapeutic agents, A24 does not affect cell cycle but induces cell apoptosis through an intrinsic pathway as assessed by caspase-9 and caspase-3 cleavage. In addition to chemotherapy, immunotherapeutic agents, such as rituximab, have raised hope for improved efficacy. Here, whereas rituximab has only a marginal effect on MCL proliferation in vitro, A24 has a high inhibiting capacity (~85% inhibition at 7.5 nmol/L, whereas rituximab reaches only ~25%). In addition, whereas rituximab mainly relies on ADCC, A24 directly acts on target cells independently of complement, of antibody aggregation, or of immune cell activity. Therefore, A24 could be also a complement to chemotherapy and to other immunotherapeutic agents in first-line treatments.

A24 but not the anti-CD20 antibody (rituximab) induces receptor endocytosis in MCL cells. Down-modulation of TfR expression by A24 is rapid, reaching 50% within 30 min of receptor engagement. AP-2 complex is the key to distinguish between two endocytic pathways, the prototypes of which being epidermal growth factor receptor and TfR, respectively. The TfR natural ligand Fe-Tf induces receptor endocytosis through an AP-2–dependent pathway. As A24 induces TfR endocytosis in a nonphysiologic setting, we questioned whether this triggering is dependent on the classic AP-2–dependent mechanism. A24-induced endocytosis was abrogated in AP-2–deficient cells, suggesting that binding of either Fe-Tf or A24 induced similar initial steps for receptor endocytosis.
However, subcellular location of TIR by confocal microscopy showed that A24 targets directly receptors of LAMP-1 and LAMP-2–positive lysosomal compartments within 15 min of incubation and impairs its recycling to the cell surface. Thus, engagement by A24 induces TIR sequestration in lysosomal compartments. Whereas the TIR recycling pathway is well characterized, the mechanism involved in its degradation remains to be established.

The comparative analysis of receptor internalization induced by A24 and transferrin could help understand this still unresolved question and to identify the adapter proteins implicated in its degradation pathway. We conclude that A24-induced switching of TIR internalization from recycling vesicles to a lysosomal compartment would affect the iron supply of MCL cells, thus favoring their apoptosis.

In summary, our study provides evidence that treatments based on the anti-TIR mAb A24 are expected to have strong potential to prevent relapse and to complement current first-line therapies in MCL.

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Prevention of Mantle Lymphoma Tumor Establishment by Routing Transferrin Receptor toward Lysosomal Compartments

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