Rapamycin Reverses Splenomegaly and Inhibits Tumor Development in a Transgenic Model of Epstein-Barr Virus–Related Burkitt’s Lymphoma

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Abstract

Epstein-Barr virus (EBV) infection and latency has been associated with malignancies, including nasopharyngeal carcinoma and Burkitt’s lymphoma. EBV encoded latent membrane protein 2A (LMP2A) is expressed in most EBV-associated malignancies and as such provides a therapeutic target. Burkitt’s lymphoma is a hematopoietic cancer associated with the translocation of c-MYC to one of the immunoglobulin gene promoters leading to abnormally high expression of MYC and development of lymphoma. Our laboratory has developed a murine model of EBV-associated Burkitt’s lymphoma by crossing LMP2A transgenic mice with MYC transgenic mice. Since LMP2A has been shown to activate the PI3K/Akt/mTOR pathway, we tested the therapeutic efficacy of mTOR inhibitor rapamycin on the tumors and splenomegaly in these double transgenic mice (Tg6/λ-MYC). We found that rapamycin reversed splenomegaly in Tg6/λ-MYC mice prior to tumor formation by targeting B cells. In a tumor transfer model, we also found that rapamycin significantly decreased tumor growth, splenomegaly, and metastasis of tumor cells in the bone marrow of tumor recipients. Our data show that rapamycin may be a valuable candidate for the development of a treatment modality for EBV-positive lymphomas, such as Burkitt’s lymphoma, and more importantly, provides a basis to develop inhibitors that specifically target viral gene function in tumor cells that depend on LMP2A signaling for survival and/or growth. Mol Cancer Ther; 10(4); 679–86. ©2011 AACR.

Introduction

Epstein-Barr virus (EBV) is a ubiquitous virus infecting oral epithelial cells and B lymphocytes (1). Infection in early childhood typically leads to an asymptomatic infection whereas infection in adolescence or adulthood can result in infectious mononucleosis (2). Primary infection is mostly cleared by a T-cell response; but, a latent infection is established in a very few infected B cells (1). EBV latency has been associated with malignant diseases including nasopharyngeal carcinoma, Hodgkin’s lymphoma, Burkitt’s lymphoma, and post-transplant lymphoproliferative disorders (PTLD; ref. 1). In addition, HIV/AIDS patients are particularly susceptible to EBV-related malignancies. Latent membrane protein 2A (LMP2A) is an EBV-encoded protein that functions as a B-cell receptor (BCR) mimic (3–5). It contains WW motifs, which mediate association with Nedd4 family ubiquitin ligases, resulting in degradation of cellular proteins (6–9). LMP2A also contains an immunoreceptor tyrosine-based activation motif that the Syk tyrosine kinase binds and a related phosphotyrosine motif that the Lyn kinase binds (3, 10, 11). As a result, LMP2A induces a BCR-like signal even in the absence of a functional BCR (3, 5). Finally, studies have shown that LMP2A is expressed in primary Burkitt’s lymphoma biopsies using a combination of PCR and Western blotting (12–15).

Burkitt’s lymphoma is a hematopoietic cancer associated with the translocation of c-MYC to one of the immunoglobulin gene promoters leading to abnormally high expression of MYC and development of lymphoma (16). Transgenic expression of MYC in mice leads to lymphoma development (17, 18). Our laboratory has developed a model of EBV-associated Burkitt’s lymphoma (Tg6/λ-MYC) by crossing LMP2A transgenic mice (Tg6) with MYC transgenic mice (λ-MYC; refs. 4, 5, 17, 19). The Tg6/λ-MYC mice develop lymphoma around 6 weeks of age whereas the λ-MYC mice typically take greater than 25 weeks to develop lymphoma (19, 20).

The activation of PI3K/Akt/mTOR pathway has been observed in many EBV-associated disorders (21). Earlier studies have shown that Akt is a downstream target of LMP2A in B cells (22–25). LMP2A expression induces phosphoinositide 3-kinase (PI3K)-dependent AKT phosphorylation (22, 26) and activation of rapamycin-sensitive...
mTOR pathway (27). Rapamycin inhibits cell growth and modulates the cell cycle of EBV-positive lymphomas in vitro (27, 28). Therefore, we tested the effect of mTOR inhibitor rapamycin on the splenomegaly and tumor development in the Tg6/\(\lambda\)-MYC mice. We found that rapamycin reversed spleen enlargement (splenomegaly) and decreased tumor size in both pretumor and lymphoma transfer models indicating that rapamycin blocks LMP2A-activated pathways that promote cell survival and tumor development.

Materials and Methods

Animals

All animal experiments were conducted in the animal facility of the Center for Comparative Medicine at the Northwestern University following Institutional Animal Care and Use Committee guidelines. Wild-type C57BL/6 and Rag1 KO (knockout; B6.129S7-Rag1\(^{tmMom}\)/J, catalogue no. 002216) mice were purchased from Jackson Laboratories. The MYC transgenic (\(\lambda\)-myc) and LMP2A–MYC double transgenic (Tg6/\(\lambda\)-MYC) mice have previously been described (5, 17, 19, 29).

Tumor transfer model

Cervical or peripheral lymph node tumors were harvested from \(\lambda\)-MYC or Tg6/\(\lambda\)-MYC mice, processed into single cells, and either immediately used or aliquoted and frozen at 140°C. For tumor transfer, the freshly isolated or thawed \(1 \times 10^6\ \lambda\)-MYC or Tg6/\(\lambda\)-MYC lymphoma cells were subcutaneously implanted into the right flank of anesthetized Rag1 KO mice which developed local tumors in 2 to 3 weeks.

Rapamycin treatment

Rapamycin (LC Laboratories) was dissolved in dimethyl sulfoxide (DMSO) at 45 mg/mL, aliquoted, and kept at −20°C during the study. On the treatment day, the aliquots were diluted with 5.1% polyethylene glycol (PEG-400; EMD, Fisher) and 5.1% Tween-80 (Fisher) immediately before use as previously reported (30). In both pretumor and tumor transfer models (when tumors were palpable), the animals received 5 mg/kg of rapamycin or equivalent amount of DMSO as intraperitoneal injections daily for 10 days. On day 11, the animals were sacrificed; tumor, spleen, and bone marrow were harvested, documented, and analyzed with flow cytometry. In the pretumor model, the mice were 5 to 9 weeks old when the treatment was started; the data are from 1 of 3 separate experiments, and each data point is from 2 to 6 mice. In the tumor transfer model, mice were 8 to 15 weeks old at the time of tumor cell transfer. The data shown are from 1 of 2 separate experiments. Each data point is from 3 to 5 mice.

Flow cytometry

The harvested tumors, spleens, or bone marrow were processed into single cells. One million cells were stained with specific antibodies, acquired with FacsCantorII (BD Biosciences) at the Northwestern University Immunobiology Flow Cytometry Core Facility. The following antibodies were used to stain the cells: B220-V450, CD19-FTTC, IgM-PE, 7-AAD, Annexin V-APC (BD Biosciences), and CD3 (eBioscience). The data were analyzed with FlowJo software (Tree Star). Unless otherwise indicated, the following sequential gating was done for analysis: live cells (7AAD negative), singlet, lymphocyte, and population of interest.

Statistical analysis

The data were analyzed with unpaired 2-tailed t test using GraphPad Prism (GraphPad Software Inc.). Data were graphed in a box-and-whisker format. The box for each group represents the interquartile range (25th–75th percentiles) and the longer line in the box is the median value. The mean is indicated by a + sign, which may be seen as a short line when it coincides with a vertical line or absent when the median and mean correspond. The whiskers indicate minimum and maximum data points. Note that when there are less than 4 data points in a group, a vertical line is shown and not a box. The P value of 0.05 and below was considered statistically significant.

Results

Rapamycin reverses splenomegaly in Tg6/\(\lambda\)-MYC mice

To determine whether rapamycin treatment inhibits LMP2A-induced splenomegaly, wild-type, \(\lambda\)-MYC, or Tg6/\(\lambda\)-MYC mice were treated daily with 5 mg/kg of rapamycin or equivalent amount of DMSO for 10 days. Although rapamycin treatment did not significantly alter the size of the spleen in either wild-type or \(\lambda\)-MYC mice, it decreased the spleen size in Tg6/\(\lambda\)-MYC lymphoma cells subcutaneously implanted into the right flank of anesthetized Rag1 KO mice which developed local tumors in 2 to 3 weeks.
early age, have a dramatic decrease in the CD3-positive population in the spleen. After tumor development, this number further decreases to below 1% (data not shown). Rapamycin treatment increased the CD3-positive cell population from 0.7% to 25% nearly to that of wild type. The percentage of CD3 population in wild type or \(\lambda\)-MYC did not change with rapamycin treatment (Fig. 1D).

**Tg6/\(\lambda\)-MYC lymphoma cells form tumors in recipient Rag1 KO mice**

To test the effect of candidate therapeutic compounds that may specifically target Tg6/MYC lymphomas, we established a tumor transfer model, in which we implanted \(1 \times 10^6\) \(\lambda\)-MYC or Tg6/\(\lambda\)-MYC lymphoma cells subcutaneously into the right flank of Rag1 deficient (Rag1 KO) mice and observed for tumor development. The kinetics of secondary tumor development was very similar in the recipients and tumors developed in both groups within 3 weeks after the cell transfer (data not shown).

**Rapamycin decreases Tg6/\(\lambda\)-MYC tumor mass in recipient Rag1 KO mice**

To test the effect of the rapamycin on LMP2A-associated tumor development, we treated recipient mice in our tumor transfer model with rapamycin after tumors were palpable. Rapamycin treatment significantly inhibited tumor development as well as splenomegaly in Tg6/\(\lambda\)-MYC tumor recipients (Fig. 2). The tumor mass was significantly reduced in recipients of both Tg6/\(\lambda\)-MYC and \(\lambda\)-MYC tumors after rapamycin treatment; however, this decrease was much more pronounced in the recipients of Tg6/\(\lambda\)-MYC tumor cells than in those that received \(\lambda\)-MYC tumor cells (Fig. 2A and C). In mice receiving Tg6/\(\lambda\)-MYC lymphoma cells, the average tumor mass was 5.09 g (SEM = 0.508) in the DMSO control group, whereas it was 1.04 g (SEM = 0.229) in the rapamycin-treated group (Fig. 2C). This decrease corresponds to an 80% reduction \((P = 0.0001)\). The rapamycin-induced decrease in the tumor size was less pronounced in mice receiving \(\lambda\)-MYC lymphoma cells.
The tumor size in the \(\lambda\)-MYC lymphoma recipients was 2.25 g (SEM = 0.46) in the DMSO group and 0.94 g (SEM = 0.27) in the rapamycin-treated group (Fig. 2C). This decrease corresponds to a 58\% inhibition (\(P = 0.0476\)). We also observed that the Tg6/\(\lambda\)-MYC tumors generally grew to a larger size than \(\lambda\)-MYC tumors in the recipients (Fig. 2A and C), which may be attributed to proliferation and survival signals induced by LMP2A and reflective of the much more rapid onset of these tumors when compared to the \(\lambda\)-MYC tumors.

Rapamycin decreases Tg6/\(\lambda\)-MYC–induced splenomegaly in recipient Rag1 KO mice

In our tumor transfer model, despite the fact that the tumor is implanted subcutaneously into the right flank of the mice, the tumor cells metastasize into other organs including bone marrow and spleen. One result of this dissemination is the development of splenomegaly in the recipients. With the rapamycin treatment, we observed a significant decrease in the splenomegaly only in Tg6/\(\lambda\)-MYC tumor recipients (Fig. 2B and D). In the mice receiving Tg6/\(\lambda\)-MYC lymphoma cells, the average spleen mass was 413 mg in the DMSO control group, whereas it was 208 mg in the rapamycin group (Fig. 2D). This decrease corresponds to a 50\% inhibition of splenomegaly (\(P = 0.0041\)). In the \(\lambda\)-MYC tumor recipients, there was no significant difference between the 2 groups (Fig. 2D). The spleen size in the \(\lambda\)-MYC lymphoma recipients was 677 mg in the DMSO group and 635 mg in the rapamycin group (Fig. 2D). This corresponds only to a 6\% inhibition (\(P = 0.819\)). In addition, we also observed that the spleens in recipients of Tg6/\(\lambda\)-MYC lymphoma cells were generally smaller than those of mice that received \(\lambda\)-MYC lymphoma cells (Fig. 2B and D).

Rapamycin decreases metastasis of Tg6/\(\lambda\)-MYC lymphoma cells into the bone marrow of recipient Rag1 KO mice

In preliminary experiments, we had observed that the transferred lymphoma cells repopulated the recipient bone marrow. To assess whether rapamycin treatment was also effective in decreasing the metastases of lymphoma cells, we tested for B cells using the pan B-cell marker CD19 in the bone marrow of recipient mice. Rag1 KO mice do not have mature B cells (31). In the naive Rag1 KO, we detected a small number (3\%) of CD19-positive cells in the bone marrow, which may correspond to early B-cell progenitors. In wild-type mice, approximately 30\% of the cells were CD19-positive B cells. In the tumor-transferred Rag1 KO mice, however, we observed a very high percentage of CD19-positive cells in the bone marrow. The increased percentage of CD19-positive bone marrow cells is indicative of metastasis of transferred lymphoma cells into the bone marrow. In rapamycin-treated group, the percentage of CD19-positive cells in the bone marrow significantly decreased in the Tg6/\(\lambda\)-MYC group (Fig. 3, left). The percentage of CD19-positive cells in the bone marrow of Tg6/\(\lambda\)-MYC recipients was 45\% and 18\% in DMSO and rapamycin groups, respectively. This corresponds to a 60\% decrease (\(P = 0.012\)). Even though there was a considerable decrease in the percentage of CD19-positive cells in the bone marrow of \(\lambda\)-MYC tumor recipients treated with rapamycin, this difference did not reach statistical significance. The percentages of CD19-positive cells in the bone marrow of \(\lambda\)-MYC recipients were 68\% and 42\% in DMSO and rapamycin groups, respectively (Fig. 3, left). This corresponds to a 38\% decrease (\(P = 0.209\)).

As in the bone marrow, there were only a few percent of, if any, CD19-positive cells in the spleen of naive Rag1 KO mice. However, in the spleen of lymphoma recipients,
more than 70% of splenocytes were CD19 positive. After rapamycin treatment, we observed a slight decrease in the percentage of CD19-positive cells in the spleens of both groups (Fig. 3, middle). In the Tg6/λ-MYC recipients, the average percentage of CD19-positive cells in the spleen was 78% and 70% in DMSO and rapamycin groups, respectively. Similarly, in the recipients of λ-MYC lymphoma cells, the corresponding values were 90% and 81% in DMSO and rapamycin groups, respectively.

Almost all the tumor cells (92%–99%) in recipients of both Tg6/λ-MYC and λ-MYC lymphoma cells were CD19 positive (Fig. 3, right). Rapamycin treatment caused a slight but statistically significant decrease in CD19-positive cells in the tumors of Tg6/λ-MYC lymphoma recipients (from 99% to 95%; P = 0.030). The decrease in the corresponding CD19-positive values in the λ-MYC lymphoma recipients, however, was not significant (from 97% to 96%; P = 0.553). In addition, the percentages of CD19-positive cells in the bone marrow and spleen, but not in the tumor, of the λ-MYC tumor recipients were slightly higher than those of Tg6/λ-MYC recipients (Fig. 3).

**Rapamycin induces apoptosis of Tg6/λ-MYC lymphoma cells in the spleen of recipients**

To assess whether the decrease in the tumor size and splenomegaly correlated with increased cell death, we measured apoptosis by Annexin V staining. Figure 4A shows Annexin V staining of live cells (7AAD negative) in representative samples from DMSO or rapamycin groups of Tg6/λ-MYC and λ-MYC lymphoma recipients. Overall, the rapamycin treatment caused an increase in apoptosis in total live splenocytes and tumor cells (data not shown). To assess the specific effect of rapamycin on tumor cells, we then analyzed the percentage of Annexin V-positive cells on live, CD19-positive gated cells (Fig. 4B). In the recipients of Tg6/λ-MYC lymphoma cells, we noticed a significant increase in the level of apoptosis in CD19-positive cells from the spleen (Fig. 4B, middle, from 18% to 27%, P = 0.022) but a
significant decrease in apoptosis in the bone marrow CD19-positive cells (Fig. 4B, left, from 17% to 10%, \( P = 0.015 \)) in the same recipients. In the tumors of Tg6/\( \lambda \)-MYC recipients treated with rapamycin, there was highly variable Annexin V staining and despite the higher mean value of apoptosis (4.7% vs. 12.8%), the level did not reach statistical significance (Fig. 4B, right). In the recipients of \( \lambda \)-MYC lymphoma cells, there was no significant change in the apoptosis level in spleen, bone marrow, or tumor cells with rapamycin treatment (Fig. 4B).

**Discussion**

Our data show that rapamycin significantly interferes with the LMP2A-induced survival signaling in our transgenic Tg6/\( \lambda \)-MYC model of Burkitt’s lymphoma. Indicative of the sensitivity of Tg6/\( \lambda \)-MYC cells to rapamycin, we observed a dramatic reduction in spleen size and a restoration of normal splenic T- and B-cell populations when Tg6/\( \lambda \)-MYC mice were treated prior to tumor development (Fig. 1). This dramatic difference was not observed in control \( \lambda \)-MYC or wild-type mice. In our tumor transfer model, Tg6/\( \lambda \)-MYC tumors were very sensitive to rapamycin treatment (Figs. 2 and 3). We did observe a modest effect on \( \lambda \)-MyC tumors with rapamycin treatment, but this effect was not of statistical significance in contrast to Tg6/\( \lambda \)-MyC tumors.

Interestingly, despite the limited sensitivity of \( \lambda \)-MyC tumors to rapamycin, splenomegaly was not reversed by rapamycin following \( \lambda \)-MyC tumor transfer in contrast to when Tg6/\( \lambda \)-MyC tumor cells were transferred. The nature of this difference is not clear, but it may be related to additional changes or the splenic microenvironment that may render \( \lambda \)-MyC tumors less dependent on the mTOR pathway when the tumor has metastasized to the spleen.

We also observed increased apoptosis in Tg6/\( \lambda \)-myc tumor cells when compared with \( \lambda \)-MYC tumor cells (Fig. 4) which may be related to our previous observation that the p53 pathway is still active in the Tg6/\( \lambda \)-MYC tumors which harbor p53 pathway mutations (19, 32). The absence of p53 pathway mutations in the Tg6/\( \lambda \)-MYC tumors may result in tumor cells more susceptible to apoptotic signals such as treatment with rapamycin when compared with \( \lambda \)-MYC tumors.

Related to apoptosis sensitivity, we noted an interesting observation indicating that Tg6/\( \lambda \)-MYC tumor cells in the bone marrow were resistant to rapamycin treatment when compared with tumor cells in the spleen (Fig. 4). Rapamycin has been previously shown to induce apoptosis of cancer cells (33, 34). It has also been shown to inhibit apoptosis in human promyelocytic leukemia cell line HL-60, which may be through rapamycin-induced downregulation of FKBPI2, rapamycin’s binding partner (35). The resistance of Tg6/\( \lambda \)-MYC tumor cells to rapamycin may be related to factors present in the tumor microenvironment such as cytokines or tumor stromal cells (36, 37). Quiescent leukemic stem cells residing in the bone marrow have been shown to be resistant to the treatment and this resistance can be broken if the cells are driven into the cell cycle (38). The low percent of CD19-positive bone marrow cells in our model may be noncycling tumor stem cells and therefore resistant to the induction of apoptosis by rapamycin whereas tumor cells present in the spleen may have a more activated phenotype and as such be more susceptible to rapamycin. In addition, LMP2A has been shown to inhibit apoptosis induced by BCR and TGF-\( \beta \) (24, 39) and signaling through these receptors in different microenvironments may also play a role in the differential sensitivity of Tg6/\( \lambda \)-MYC tumor cells compared with \( \lambda \)-MYC tumor cells.

In line with our studies, there have been some reports suggesting that rapamycin can inhibit EBV-related PTLD (28, 33), whereas others have indicated insufficiency of rapamycin monotherapy in the prevention of PTLD (34, 40). This discrepancy may be related to differences in cellular and viral genes expressed but more importantly may be due to the evolution of the PTLD and the ability of cells to overcome rapamycin inhibition. In addition to inhibiting mTOR, rapamycin also inhibits the inhibitory feedback loop on the Akt activation (41). While mTORC1 complex is sensitive, the mTORC2 complex is resistant to rapamycin inhibition (42, 43) and the activity of mTORC2 has been shown to be required for prostate tumor development (44). Even in the presence of rapamycin, the resistant mTORC2 complex is able to activate Akt leading to an incomplete inhibition of the PI3K/Akt/mTOR survival pathway (42, 43, 45, 46) resulting in tumor cells addicted to Akt activation. Previous studies have shown that LMP2A activates the PI3K/Akt/mTOR pathway (22, 23, 25). Therefore, more potent dual inhibitors of PI3K/Akt/mTOR pathway, such as BEZ235, p242, or Torin-1, may be more effective in inhibiting the lymphomas that occur in the Tg6/\( \lambda \)-MYC as was recently shown for KSHV tumor cells (36, 47–50).

We show that rapamycin effectively and specifically reverses the LMP2A-induced splenomegaly and significantly impairs the development of tumors expressing LMP2A. Our data support previous studies that LMP2A modulates the PI3K/Akt/mTOR pathway and that this modulation is sensitive to mTOR inhibition. Furthermore, our data indicate that rapamycin could be a valuable candidate for the development of a treatment regimen for LMP2A-expressing EBV-positive abnormalities LMP2A is thought to be expressed in most EBV-associated pathologies that occur in the human host, but the level of LMP2A expression can vary in the diverse disorders which include Burkitt’s lymphoma, Hodgkin’s lymphoma, nasopharyngeal carcinoma, HIV-associated secondary malignancies, and PTLD. How levels of LMP2A expression may impact the sensitivity of EBV-related pathologies to treatment with therapeutic agents,
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such as rapamycin, will need to be investigated. LMP2A transgenic mice with varying levels of LMP2A expression may offer an important experimental system to investigate this question.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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