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Concomitant reduction of c-Myc expression and PI3K/AKT/mTOR signaling by quercetin induces a strong cytotoxic effect against Burkitt's lymphoma

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ABSTRACT

Burkitt's lymphoma is an aggressive B cell lymphoma whose pathogenesis involves mainly c-Myc translocation and hyperexpression, in addition to antigen-independent BCR signaling and, in some cases, EBV infection. As result of BCR signaling activation, the PI3K/AKT/mTOR pathway results constitutively activated also in the absence of EBV, promoting cell survival and counterbalancing the pro-apoptotic function that c-Myc may also exert. In this study we found that quercetin, a bioflavonoid widely distributed in plant kingdom, reduced c-Myc expression and inhibited the PI3K/AKT/mTOR activity in BL, leading to an apoptotic cell death. We observed a higher cytotoxic effect against the EBV-negative BL cells in comparison with the positive ones, suggesting that this oncogenic gammaherpesvirus confers an additional resistance to the quercetin treatment. Besides cell survival, PI3K/AKT/mTOR pathway also regulates autophagy: we found that quercetin induced a complete autophagic flux in BL cells, that contributes to c-Myc reduction in some of these cells. Indeed, autophagy inhibition by chloroquine partially restored c-Myc expression in EBV-positive (Akata) and EBV-negative (2A8) cells that harbor c-Myc mutation. Interestingly, chloroquine did not affect the quercetin-mediated reduction of c-Myc expression in Ramos cells, that have no c-Myc mutation in the coding region, although autophagy was induced.

These results suggest that mutant c-Myc could be partially degraded through autophagy in BL cells, as previously reported for other mutant oncogenic proteins.

1. Introduction

Burkitt's lymphoma (BL) is an aggressive germinal center (GC) B cell lymphoma that exists in three distinct forms: endemic BL (eBL), sporadic BL (sBL) and AIDS-related BL. While eBL is 100% associated with the oncogenic gammaherpesvirus Epstein–Barr (EBV), the other two forms are associated with the virus in a lower percentage of cases (20–40%) (Kelly and Rickinson, 2007). As a common feature, all forms of BL display cellular myelocytomatosis oncogene (c-Myc) translocation to one of the immunoglobulin loci. In most of the cases, c-Myc is translocated to the heavy chain immunoglobulin locus and, in a smaller percentage of cases, to the light chain immunoglobulin loci. Anyway, c-Myc translocation results in its overexpression (Boxer and Dang, 2001). Although typical of BL, c-Myc translocation can in some cases occur in other hematological tumors such as diffuse large cell lymphoma (DLBL) and multiple myeloma (Schmitz et al., 2014, 2012) (Seidl et al., 2003). In addition to translocation, c-Myc can often undergo point mutations in BL, the most common of which occurs at Threonine 58 (Thr-58). This is a phosphorylation site essential for c-Myc proteasomal degradation (Bahram et al., 2000). Other mutations could occur at Serine 62

Abbreviations: Q, quercetin; BL, Burkitt's lymphoma; BCR, B cell receptor; GC, Germinal Center; EBV, Epstein–Barr virus; PI3K, phosphatidylinositol-3-kinase; AKT, AKT or protein kinase B; mTOR, mammalian target of rapamycin; BEZ235, NVP-BEZ235; I-c-Myc, c-Myc inhibitor; HSPs, heat shock proteins; PES, 2-phenylethynesulfonamide; CQ, chloroquine; PARP, poly (ADP-ribose) polymerase; LC3, microtubule-associated protein 1A/1B-light chain 3; 4E-BP1, eIF4E-binding protein; c-Myc, cellular myelocytomatosis oncogene; NF-κB, nuclear factor K-light-chain-enhancer of activated B cells; Bcl-2, B-cell lymphoma 2; TCF-3, transcription factor 3; GSK3, glycogen synthase kinase 3; PES, 2-phenylethyne-1-sulfonamide, 2-phenylethynesulfonamide.

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(Ser-62), resulting in opposite effect since phosphorylation in this site stabilizes c-Myc (Junttila and Westermarck, 2008). However, as Ser-62 phosphorylation is a prerequisite for c-Myc phosphorylation at Thr-58 (Gregory et al., 2003), mutations both at Thr-58 and Ser-62 influence c-Myc stabilization and likely its transcriptional activity. c-Myc point mutations can also occur in sites that alter c-Myc functions, such as the transactivation of the pro-apoptotic B-cell lymphoma 2 (Bcl-2) family member Bim, resulting in a reduction of its activity (Herbst et al., 2005). c-Myc plays an essential role in the pathogenesis of BL, to which also contributes an aberrantly activated phosphatidylinositol-3-Kinase/AKT or PKB/mammalian target of Rapamycin (PI3K/AKT/mTOR) signaling. Indeed, mice model engineered to hyperexpress c-Myc and a constitutively active form of PI3K in B cells undergoing the GC reaction, develop an aggressive lymphoma that strongly resembles BL (Sander and Rajewsky, 2012).

PI3K activation in human BL can be also induced by Epstein–Barr virus (EBV) (Chen, 2012). However, the activation of this pathway is mainly mediated by a constitutive "tonic" B cell receptor (BCR) signaling, which occurs independently of antigen stimulation and, therefore, it does not involve the activation of the nuclear factor K-light-chain-enhancer of activated B cells (NF-κB) pathway. PI3K activation in BL is also positively influenced by Transcription Factor 3 (TCF-3), a constitutively activated transcription factor that augments the BCR signaling (Schmitz et al., 2014, 2012). Interestingly, c-Myc may contribute to the activation of PI3K pathway by promoting the expression of miR-17-92 cluster (Olive et al., 2009; Rao et al., 2012; Xiao et al., 2008) and, on the other hand, the PI3K pathway controls c-Myc expression level through Glycogen Synthase Kinase 3 (GSK3) that mediates c-Myc phosphorylation at Thr-58 (Gregory et al., 2003). Moreover, PI3K activation has been reported to control c-Myc stability (Kumar et al., 2006) also through an increase of expression or function of HSP70 and HSP90 (Chatterjee et al., 2013), of which c-Myc is a client protein (Yamaki et al., 2011). Quercetin, a flavonoid widely distributed in vegetables and beverages daily consumed (Nijveldt et al., 2001), has been reported to inhibit the PI3K/AKT/mTOR pathway (Gulati et al., 2006) and function also as a dual mTOR and PI3K specific inhibitor (Bruning, 2013). As result of this pathway inhibition, quercetin reduces growth of cancer cells with a constitutively activated PI3K pathway (Gulati et al., 2006). Importantly, quercetin may also prevent tumorigenesis (Gibellini et al., 2011).

In this study, we investigated whether quercetin could target two of the main oncogenic mechanisms involved in BL pathogenesis, namely the activation of PI3K/AKT/mTOR signaling and the hyperexpression of c-Myc. The outcome of quercetin treatment on BL cell death and autophagy was then investigated.

2. Materials and methods

2.1. Cells

Raji (EBV-positive cell line derived from Burkitt's lymphoma harboring a defective viral genome), Akata (EBV-positive cell line derived from a Burkitt's lymphoma) (Takada et al., 1991), 2A8 (EBV-negative Akata), Ramos (EBV-negative cell line derived from Burkitt's lymphoma), and BL41 (EBV-negative Burkitt's lymphoma cells) (Ramqvist et al., 1993) were cultured in RPMI 1640 (Corning, Manassas, VA, USA; R0883), 10% Fetal Bovine Serum (FBS) (Corning, Manassas, VA, USA; 35-079-CV), L-glutamine (Aurogene, Rome, Italy; AU-X0550) and streptomycin (100 μg/ml) and penicillin (100U/ml) (Aurogene, Rome, Italy; AU-L0022) in 5% CO_2 at 37 °C.

2.2. Cell treatments

Cell lines were treated with quercetin (Sigma Aldrich, St. Louis, MO, USA; Q4951) at the indicated doses for 24 h. Cells were also treated with NVP-BEZ235 (BEZ235) (100 nM) (Selleckchem, Munich, Germany; S1009), a dual inhibitor of PI3 K and mTOR protein kinases, and with c-Myc Inhibitor (I-c-Myc) (100 μM) (Millipore, Billerica, MA, USA; 475956). 2-phenylethyne-1-sulfonamide, 2-phenylethynesulfonamide (PES), the inhibitor of HSP70, (Calbiochem, San Diego, CA, USA; cat. no. 506155) was used at 20 and 30 μM for 24 h.

In order to investigate autophagy, Akata, 2A8 and Ramos cell lines were cultured in a medium containing both quercetin (100 μM) and chloroquine (CQ) (Klionsky et al.) (10 μM), a lysosomotropic agent that prevents endosomal acidification (Sigma Aldrich, St. Louis, MO, USA; C6628).

2.3. Antibodies

In this work we used the following primary antibodies: rabbit polyclonal anti-poly (ADP-ribose) polymerase (PARP) (1:500) (Cell Signaling, 9542), rabbit polyclonal anti-phospho-70S6K (Thr389) (1:300) (Cell Signaling, Davers, MA. USA; 9205), rabbit polyclonal anti-70S6K (1:500) (Cell Signaling, Davers, MA, USA; 2708) rabbit polyclonal anti-phospho eIF4E-binding protein (4E-BP1) (Thr37/46) (1:100) (Cell Signaling, Davers, MA, USA; 2855), rabbit polyclonal anti-4E-BP1 (1:300) (Cell Signaling, Davers, MA, USA; 9452), rabbit polyclonal anti-c-Myc (1:500) (Cell Signaling, Davers, MA, USA; 5605), rabbit polyclonal anti-HSP70 (1:100) (Santa Cruz Biotechnology, Heidelberg, Germany; sc-66049).

To study autophagy we used rabbit polyclonal anti-microtubule-associated protein 1A/1B-light chain 3 (LC3) (1:1000) (Novus Biologicals, Littleton, CO, USA; NB100-2220). Monoclonal mouse anti-β-actin (1:10000) (Santa Cruz Biotechnology Inc., Heidelberg; sc-137179) was used as a marker of equal loading. Goat polyclonal anti-mouse IgG-HRP (Santa Cruz Biotechnology Inc., Heidelberg; sc-2005) and anti-rabbit IgG-HRP (Santa Cruz Biotechnology Inc., Heidelberg; sc-2004) were used as secondary antibodies. All the primary and secondary antibodies used in this study were diluted in a PBS- 0.1% Tween 20 solution containing 3% BSA.

2.4. Western blot analysis

Cells (1×10^6) were washed twice with PBS solution and centrifuged at 1500 rpm for 5 min. The pellet was lysed in a RIPA buffer containing 150 mM NaCl, 1% NP-40, 50 mM Tris-HCl (pH 8), 0.5% deoxycholic acid, 0.1% SDS, protease and phosphatase inhibitors. Then, protein lysates were subjected to electrophoresis on 4–12% NuPage Bis-Tris gels (Thermo Fisher Scientific, Waltham, MA USA; N00322BOX) according to the manufacturer's instruction. Then, the gels were transferred to Nitrocellulose Membranes (Biorad, Hercules, CA, USA; 162-0115) for 2 h in Tris-Glycine. The membranes were blocked in PBS-0.1% Tween20 solution containing 3% of BSA, probed with specific antibodies and developed using ECL Blotting Substrate (Advansta, Menlo Park, CA,'USA; K-12045-D20).

2.5. Cell viability

To evaluate the cell viability, cells were plated in 12-well plates at a density of 8×10^5 cells/ml and treated with quercetin (100 μM), c-Myc Inhibitor (I-c-Myc), or NVP-BEZ235 (BEZ235) (100 nM) alone or in combination for 24 h. Akata and BL41 cells were pre-treated with z-VAD.fmk (Calbiochem, San Diego, CA, USA; 219011) (100 μM), caspase inhibitor, for 30 min and cultured with quercetin (100 μM) for 24 h. Then, a trypan blue (Sigma Aldrich,