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Cytotoxic effect caspase activation dependent of a genetically engineered fusion protein with a CD154 peptide mimetic (OmpC-CD154_p) on B-NHL cell lines is mediated by the inhibition of bcl-6 and YY1 through MAPK p38 activation

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ABSTRACT

The interaction between CD40, and its ligand, CD154, is essential for the development of humoral and cellular immune responses. The selective inhibition or activation of this pathway forms the basis for the development of new therapeutics against immunologically based diseases and malignancies. We are developing a gene fusion of *Salmonella typhi* OmpC protein expressing the CD154 Tyr140-Ser-149 amino acid strand. This OmpC-CD154 binds CD40 and activates B cells. In this study, we demonstrate that OmpC-CD154_p treatment inhibits cell growth, proliferation and induced apoptosis in the B-NHL cell lines Raji and Ramos. The Bcl-2 family proteins were regulated and the Bcl-6 and YY1 oncoproteins were inhibited. p38 MAPK activation is an important mechanism underlying the effect on proliferation and apoptosis mediated by this fusion protein. This study establishes a basis for the possible use of fusion protein OmpC-CD154 as an alternative treatment for B-NHL.

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Introduction

Non-Hodgkin lymphomas (NHLs) are lymphoid neoplasms with a B or T phenotype; B cell lymphomas are more common; NHLs represent the fourth most common cancer and the sixth most common cause of cancer death in the United States and are among the top 10 causes of cancer in both sexes worldwide [1]. The standard treatment for NHL is mainly based on the use of chemotherapy consisting of CHOP (cyclophosphamide + doxorubicin + vincristine + prednisone) in combination with the anti-CD20 monoclonal antibody Rituximab, R-CHOP has become the gold standard treatment of NHL. Although this combination represents significant progress in lymphoma treatment, 40% of patients do not respond or are refractory to this treatment. Thus, the search for new therapeutic targets and immunotherapy alternatives to improve the treatment of NHL has intensified [2].

CD40, which is a type I glycoprotein of the tumor necrosis factor (TNF) receptor superfamily, is among

the main therapeutic targets for lymphomas. CD154, which is a type II glycoprotein that is also a member of the TNF family, forms a homotrimer to which CD40 binds; each monomer of CD154 interacts with one molecule of CD40; thus, each trimer of CD154 is able to bind three molecules of CD40 [3,4]. An advantage of using CD154 in therapy is that its effect is not only achieved through complement activation and antibody-dependent cellular cytotoxicity; CD154 also forms a trimeric, or at least a dimeric, interaction to signal through CD40 to induce tumor cell apoptosis in the absence of a first signal [5,6]. Artificially inducing this signal has been a challenge due to the lack of effective strategies for trimerizing the recombinant protein or binding antibodies with leucine zippers, resulting in the inability to correctly mimic the natural function of the ligand. Therefore, proteins that trimerize naturally and fuse ligand sequences are used to recognize CD40 and form stable trimers, as is normally the case between CD40 and CD154 [7].

Therefore, we propose that the fusion protein OmpC-CD154 is a possible therapy for NHL. The OmpC protein from *Salmonella typhi* has a trimeric structure that is highly resistant to the effects of proteases and detergents. OmpC can be modified without altering its structure, allowing for the integration of the amino acid sequence of CD154, which is a protein that can activate normal B cells [8] and, due to its trimeric structure, can induce apoptosis in NHL cell lines by activating CD40. The present study was performed to test this hypothesis, and the following areas were investigated: (1) the effect of fusion protein OmpC-CD154 on proliferation and apoptosis in several B-NHL cell lines, (2) the signaling mechanisms by which the fusion protein induces proliferation and apoptosis, and (3) the effect of OmpC-CD154 on the Bcl-6 and YY1 oncoproteins.

Material and methods

Cell lines

NHL-B cell lines Raji, Ramos, Daudi, DHL4 and 2F7 were obtained from the American Type Culture Collection (ATCC, Manassas, VA).

Reagents

The Outer Membrane Protein C (OmpC) from *Salmonella typhi*, was obtained and inserted into the PST13 vector as described previously [8]. The fusion protein OmpC-CD154, which is a homotrimer with 347 amino acids from *Salmonella typhi* OmpC and 10 amino acids from CD154 (W140-S149) inserted into loop 5, was obtained as described previously [8].

CD40 Lrh contains the extracellular domain of human CD40L (CD154) (aa116-261) conjugated to a linker peptide (6 aa) and a FLAG®-tag (Enzo Life Sciences, New York, NY).

The specific p38 MAPK inhibitor LY-2228820 was obtained from Selleckchem (Houston, TX).

Obtaining and purifying OmpC and OmpC-CD154

Proteins OmpC and OmpC-CD154 were obtained as previously reported [8]. Briefly, the *Escherichia coli* strains DH5α (Invitrogen Life Technologies, Carlsbad, CA) ϕ F80dlacZΔM15Δ [(lacZYA-argF) U169 deoR recA1 end A1 hsdR17 (r⁺, m) phoA supE44 thi-1 gyrA96 relA1] and UH302 [OmpC⁻, OmpF⁻] were used. The bacterial strains were transformed with the PST13 and PST13-CD154 plasmids. The PST13-CD154 vector contains a substitution in the region encoding loop 5 of

porin OmpC with the sequence encoding the peptides Tyr140-Ser149 of CD154 [8]. The OmpC and OmpC-CD154 proteins were purified using a previously described method [8].

Determination of CD40 by flow cytometry

To identify cell surface receptors, such as CD40, the cell lines were cultured at a density of 3×10^5 cells/well in 1 mL of culture medium. Cells were incubated with an anti-Human-CD40-PE-HB14 antibody (BioLegend, San Diego, CA) for 20 min on ice. Then, the cells were recorded using a FACSVantage SE System cytometer (Becton Dickinson, Mountain View, CA) and analyzed with Lysis II software (Version 1.1) to measure the percentage of positively stained cells. The mean fluorescence intensity of CD40 was plotted.

Determination of the inhibition of cell proliferation

The inhibition of proliferation was determined using a colorimetric Cell Proliferation Kit (XTT) (Roche, USA). The results were obtained using an ELISA plate reader Multiskan FC ELISA reader (Thermo Scientific, Waltham, MA), following the manufacturer's recommendations.

Cell viability determination

The cell viability was determined using cell lines treated with the OmpC-CD154 protein by trypan blue staining (Invitrogen). The cell lines were cultured at a density of 1×10^6 cells/well in 1 mL of culture medium in 12-well, flat-bottom cell culture microplates. The treated cells were incubated with the OmpC, OmpC-CD154 or CD154rh proteins at a concentration of 2 μg/mL for 12, 18, 36 or 48 h at 37 °C in a 5% CO₂ incubator. The percentage of viable cells was determined using a Bio-Rad TC20 cell counter (Bio-Rad, Hercules CA).

Mitochondrial membrane potential analysis

The cell lines were cultured at a density of 3×10^5 cells/well in 2 mL of culture medium. Cells treated with the OmpC, OmpC-CD154 or CD154rh proteins at a concentration of 2 μg/mL were incubated for 24 h at 37 °C in a 5% CO₂ incubator. The cells were placed in microtubes. Each tube contained 100 μL of medium with 300 nM TMRE (tetramethylrhodamine ethyl ester) (Abcam, UK). The cells were incubated for

20 min at 37 °C. The mitochondrial membrane potential was analyzed by a fluorescence microplate reader (Perkin, Elmer, MA) at Ex/Em = 549/575 nm and fluorescence microscopy (Olympus, Tokyo, Japan).

Determination of signaling molecules by a Western blot analysis

The cell lines were cultured at a density of 1×10^6 cells/well in 2 mL of culture medium. Cells were incubated with the OmpC, OmpC-CD154 or CD154rh proteins at a concentration of 2 µg/mL for 24 h at 37 °C in a 5% CO₂ incubator and placed in microtubes, and 20–40 µL of M-PER Lysis Buffer were added. The cell lysates were quantified with a spectrophotometer using the Lowry method. Then, 12% acrylamide gels were prepared for an electrophoretic shift in a mini-blot chamber (Bio-Rad) with tris-glycine-SDS buffer (Bio-Rad). The membranes were blocked using Odyssey Blocking Buffer (PBS) (LI-COR, Lincoln, NE) for 1 h under constant agitation. The membranes were incubated with primary antibodies against the proapoptotic proteins Bak (Cell Signaling, Danvers, MA), Bax, PUMA and NOXA (Santa Cruz Biotechnology, Dallas TX); the antiapoptotic proteins Bcl-2 and Bcl-XL (Santa Cruz Biotechnology, Dallas, TX.); the repressor proteins Bcl-6 and YY1 (Santa Cruz Biotechnology, Dallas, TX); and p-P38 MAPK (Cell Signaling) overnight with constant agitation at 4 °C.

The membranes were washed and incubated with the anti-rabbit 680CW or anti-mouse IRDye 800CW (LI-COR, Lincoln, NE) secondary antibodies for 1 h and washed. The bands were detected by fluorescence using an Odyssey reader (LI-COR, Lincoln, NE).

Results

Heterogeneous expression of CD40 and its relationship with the effectiveness of OmpC-CD154 treatment

We analyzed the CD40 expression in several B-NHL cell lines by flow cytometry and found that CD40 was most highly expressed in the Raji cell line, while the lowest CD40 expression was observed in the Ramos cell line (Figure 1(A)) (** $p < .001$). After the expression of CD40, the effect on cell viability was analyzed. The porin OmpC native to *Salmonella typhi* was used as a negative control, and the recombinant human CD40 ligand (CD154) was used as a positive control. The treatment with the fusion protein decreased the viability of all cell lines. The greatest effect was observed in the Raji cell line, in which the fusion protein decreased the cell viability by 50.4%, whereas the lowest effect, i.e. a decrease of 22.64%, was observed in the Ramos cell line (Figure 1(B)). These data directly correlate with the CD40 expression levels in each cell line, as the treatment effect was greater in cells expressing

Figure 1. Differential CD40 expression in B-NHL cell lines and sensitivity to Fusion Protein OmpC-CD154: (A) cells (3×10^5 cells/well) were incubated with the anti-Human-CD40-PE-HB14 antibody for 20 min. The mean fluorescence intensity of three independent experiments was analyzed using Lysis II software. Two-way ANOVA statistical analysis was performed, statistical significance was considered * $p < .05$, ** for $p < .001$. (B) cell lines were cultured at a density of 1×10^6 cells/well and were incubated with the OmpC, OmpC-CD154 or CD154rh protein at a concentration of 2 µg/ml for 24 h. Then, cell viability was determined using a cell counter. The mean percent of viable cells of three independent experiments is presented on the graph. (C) Inhibition of proliferation was determined by an XTT assay. Cell lines, Ramos and (D) Raji cell lines were incubated with the OmpC, OmpC-CD154 or CD154rh protein at a concentration of 2 µg/ml for 6, 12, and 24 h at 37 °C in a 5% CO₂ incubator.

Figure 2. Treatment with OmpC-CD154 activates proapoptotic and inhibits antiapoptotic members of the Bcl-2 family: (A) Ramos and (B) Raji cell lines were cultured at 1×10^6 cells/mL and treated with 2 μ g/mL OmpC-CD154, 2 μ g/mL OmpC, or 1 μ g/mL human recombinant CD40 L plus 1 or 15 μ g/mL enhancer. The protein expression was analyzed by Western blotting.

greater amounts of CD40. Thus, we selected the Ramos and Raji cell lines for the following experiments to obtain the profiles of treatments with differing levels of effectiveness. The cellular proliferation analysis revealed that the treatment with the fusion protein decreased cell proliferation by 32% after 12 h of treatment in the Ramos cell line, whereas proliferation in the Raji cell line decreased by 55% after 12 h of treatment (Figure 1(C,D)).

Activation of proapoptotic bcl-2 family members and inhibition of Bcl-XL are OmpC-CD154-mediated apoptosis induction mechanisms

To determine the possible mechanisms by which the fusion protein OmpC-CD154 inhibits viability and proliferation in B-NHL cell lines, we analyzed the expression of Bcl-2 family proteins, particularly the proapoptotic proteins possessing the BH3 domain, after OmpC-CD154 treatment. In the Raji cells, we observed that the fusion protein increased the expression of Bak and Bax, whereas in the Ramos cells, no significant difference in their amounts was found. We also observed that the regulatory proteins PUMA and NOXA were more highly expressed compared to those in the untreated controls, but the treatment with the fusion protein decreased the expression of the antiapoptotic protein Bcl-XL in both cell lines, with a stronger effect in the Ramos cells (Figure 2). To investigate whether the treatment with the fusion protein induced apoptosis in the cell lines by destabilizing the

mitochondrial membrane potential, we performed a TMRE analysis using a fluorometer (Figure 3(A)). In the Ramos cell line, the fusion protein treatment decreased the mitochondrial membrane potential, as determined by viewing the cells under a fluorescence microscope (Figure 3(A), top column right). The same phenomenon was observed in the Raji cell line (Figure 3(A) bottom column). We also analyzed caspase activation in the Raji cell line due to its greater response to treatment. We found that caspase 3, caspase 8, and caspase 9 activation/expression increased with the treatment, whereas the expression of procaspase decreased (Figure 3(B)). Altogether, these results show that the fusion protein can induce apoptosis via the intrinsic route by activating caspase 9 and via the extrinsic route by activating caspase 8.

OmpC-CD154-mediated activation of MAPK pathways is a mechanism underlying the inhibition of proliferation and induction of apoptosis

We analyzed whether the fusion protein exerted an effect on p38 MAPK signaling and whether it decreased the expression of Bcl-6 and YY1 in B-NHL cell lines. We found that the OmpC-CD154 treatment activated p38 MAPK signaling and inhibited Bcl-6 and YY1 expression. Then, we analyzed the effect of the OmpC-CD154 treatment on Bcl-6 and YY1 in the presence of a p38 MAPK-specific inhibitor. We found that the addition of the p38 MAPK inhibitor reversed the

Figure 3. OmpC-CD154 treatment diminishes the mitochondrial membrane potential and promotes caspase activation in B-NHL cell lines. (A) top line Ramos and (A) bottom line Raji cell lines were treated with the OmpC, OmpC-CD154 or CD154rh protein at a concentration of 2 $\mu\text{g}/\text{mL}$. The mitochondrial membrane potential was analyzed by fluorescence. (B) Treatment with OmpC-CD154 promotes caspase activation Raji cells were cultured at 1×10^6 cells/mL and treated with 2 $\mu\text{g}/\text{mL}$ OmpC-CD154, 2 $\mu\text{g}/\text{mL}$ OmpC or 1 $\mu\text{g}/\text{mL}$ human recombinant CD40L plus 1 or 15 $\mu\text{g}/\text{mL}$ enhancer. (C) The protein expression was analyzed by Western blotting.

OmpC-CD154-mediated inhibition of Bcl-6 and YY1 expression. We concluded that p38 is a key element in the effect of the fusion protein and that a regulatory mechanism involving p38 expression and activation underlies this effect (Figure 4). The effect of the fusion protein diminished when the cells were treated with a specific inhibitor of p38, as observed in the XTT assays, which showed that the cells returned to their normal proliferative state after the treatment with the inhibitor. Altogether, these results demonstrate that p38 exerts a regulatory effect on Bcl-6 and YY1 (Figure 5).

Discussion

The interaction between CD40 and CD154 is highly relevant and a potential therapeutic target in the

treatment of different types of cancer. The expression of CD154 in melanoma and nasopharyngeal carcinoma is correlated with more aggressive tumor phenotypes [9–12]. CD40 is expressed at high levels in carcinomas of the cervix, ovary, bladder, and nasopharynx [13–15]. In this work, we propose using the fusion protein OmpC-CD154 as a possible alternative therapy for non-Hodgkin lymphomas.

The form of the CD40 stimulus also affects the response, since direct effects on tumor cells vary between treatments with recombinant soluble CD154 and monoclonal antibodies [16]. The difference between their effects on signaling may be related to the ability of CD40 to trimerize since the interaction between CD154 trimers and CD40 is essential for proper signaling by this pathway [17]. The original rationale for designing fusion protein OmpC-CD154

Figure 4. Inhibition of P38 MAPK abolishes the effect of OmpC-CD154. (A) Ramos and (B) Raji cell lines were cultured at 1×10^6 cells/mL or preincubated for 1 h with $10 \mu\text{M}$ p38 inhibitor (LY2228820) and then treated with $2 \mu\text{g/mL}$ OmpC-CD154, $2 \mu\text{g/mL}$ OmpC or $1 \mu\text{g/mL}$ human recombinant CD40 L plus $1 \mu\text{g/mL}$ enhancer.

was to use the trimeric structure of the OmpC protein as a carrier of a trimer of CD154 regions that can interact with and thus activate CD40 in this context. The effect of this fusion protein is dependent on its interaction with CD40 and the expression of CD40 in the target cells, as has been described by other authors [18], and as shown in our results in which the response to the induction of apoptosis and inhibition of proliferation was directly correlated with the level of CD40 expression in the cells. Notably, even if CD40 expression is low, OmpC-CD154 inhibits proliferation and induces apoptosis in all cell lines.

We analyzed the mechanism by which the fusion protein caused this phenomenon and observed that the expression of Bak and Bax, which are fundamental for the induction of apoptosis by the intrinsic

pathway, was altered. These alterations coincide with the diminished mitochondrial membrane potential and decreased Bcl-XL expression observed following the treatment with the fusion protein, facilitating the formation of mitochondrial membrane pores that trigger the induction of apoptosis together with the increased expression of the Bax activator PUMA. The OmpC-CD154 fusion protein also activated caspase 9. Caspase 3 plays an important role in the regulation of apoptosis and can be activated by caspases 9 or 8 via the intrinsic or extrinsic pathway of apoptosis, respectively [19]. The fusion protein demonstrated the ability to activate both caspases 9 and 8, highlighting that the induction of apoptosis mediated by this protein is carried out by both the intrinsic and extrinsic pathways. We suggest that the induction of apoptosis

Figure 5. (A) Ramos and (B) Raji cell lines were cultured at 1×10^6 cells/mL and preincubated with $10 \mu\text{M}$ p38 inhibitor (LY228820) for 1 h. Then, the cells were treated with $2 \mu\text{g/mL}$ OmpC-CD154, $2 \mu\text{g/mL}$ OmpC or $1 \mu\text{g/mL}$ human recombinant CD40L plus 1 or $15 \mu\text{g/mL}$ enhancer. Proliferation was determined by an XTT assay.

mediated by the chimeric protein is caspase-dependent and similar to the previously described CD40/CD154 system [20]. The results in the present suggested that the induction of apoptosis mediated by the activation of caspases is a consequence of the activation of the p38 MAPK pathway, since when the p38 inhibitor is used, the inhibition of proliferation is reversed.

OmpC-CD154 inhibited Bcl-6 expression in the NHL cell lines analyzed here. Bcl-6 expression in diffuse B cell lymphomas of the germinal center subtype is involved in the survival and proliferation of lymphoma cells [21]. Recent studies have shown that the use of an antibody against CD40 (Dacetuzumab) had antiproliferative and apoptotic effects against a panel of different B cell lines of lymphomas [22]. High levels of Bcl-6 are known to induce apoptosis and inhibit proliferation in germinal center lymphomas in which Bcl-6 is regulated by the MAPK pathway. OmpC-CD154

induces a sustained increase in p38 MAPK activation. Previous studies investigating the anti-CD40 antibody Dacetuzumab have observed the sustained activation of p38 MAPK for 48 h, which was also observed in this study with OmpC-CD154. We observed that the treatment with the specific p38 MAPK- α inhibitor LY-2228820 reestablished Bcl-6 expression, indicating that p38 plays an important role in the OmpC-CD154-mediated regulation of this protein.

Recent studies have described the binding of CCCTC binding factor (CTCF) to the regulatory region of exon A1 of Bcl-6; this binding is associated with active chromatin labels and counteracts the negative autoregulation of Bcl-6, and CTCF silencing is related to the reduction in Bcl-6 expression in germinal center cells [23]. The phosphorylation of C-terminal CTCF serine residues by cyclin-dependent kinase inhibitor protein I (CKI) interferes with the regulatory role of CTCF [24] and may be dependent on the p38 MAPK pathway [25]; however, conclusive evidence of this regulation is lacking. Battle, et al. proposed that a small region at the beginning of the first exon of Bcl-6 may be important for transcriptional regulation [26]. However, the Mxi2 isoform of p38 α interacts with ERK1/2 and has profound effects on ERK functioning by maintaining its phosphorylation levels and thus prolonging the duration of the ERK signal [27]. These data show that p38 MAPK plays an important role in the regulation of Bcl-6 and that the fusion protein OmpC-CD154 affects the inhibition of proliferation.

Yin Yang 1 protein (YY1) is a multifunctional transcription factor involved in the development and progression of several types of cancer [28].

The treatment with the fusion protein clearly inhibits YY1, which could also be associated with its effect on the induction of apoptosis. Importantly, in the signaling of the fusion protein, the inhibition of YY1 expression is correlated with the inhibition of Bcl-6 expression, and YY1 expression is reestablished once the p38 MAPK pathway is inhibited. These data establish a previously unknown relationship between Bcl-6/YY1 and the p38 MAPK pathway. A previous study analyzing Bcl-6 interacting proteins by tandem mass spectrometry has reported that YY1 interacts with Bcl-6 [29]. This work could be important for the development of new strategies for the treatment of various types of tumors involving these proteins. These results indicate that this protein is promising as a new therapeutic agent in the treatment of non-Hodgkin lymphomas that may be used in the future as a possible monotherapy, an adjuvant in treatments with immunotherapy, or a complementary treatment to chemotherapy.

Figure 6. Schematic representation illustrating the p38 MAPK-mediated inhibition of Bcl-6 and YY1 and the mechanism of apoptosis induction by the OmpC-CD154 fusion protein. Treatment with OmpC-CD154 in NHL cell lines increases the expression of the proapoptotic proteins Bak and Bax and inhibits the expression of the regulatory proteins PUMA, NOXA and Bcl-XL, which is similar to the cytotoxic mechanism of mitochondrial membrane potential disruption. Caspase 8 activation is related to the extrinsic apoptosis pathway, whereas caspase 9 is activated after cytochrome c release by the intrinsic pathway, and apoptosis concludes with the activation of caspase 3. Finally, p38 MAPK induces Bcl-6 and YY1 inhibition by a mechanism that is currently not fully understood.

The inhibition of Bcl-6 and YY1 mediated by p38 MAPK activation as a mechanism of apoptosis induction is shown in Figure 6.

Geolocation information

This work was carried out in Mexico with close collaboration with the USA.

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Data availability statement

All data generated or analyzed during this study are included in this article.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article online at <https://doi.org/10.1080/10428194.2018.1516286>.

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