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Short Communication

Telomerase activity in response to mild oxidative stress

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Abstract

We have analysed telomerase activity to determine whether it can be modified when BCL-2 is endogenously overexpressed in response to a mild oxidative stress treatment as part of a survival mechanism, in contrast with an exogenous *bcl-2* overexpression due to a retroviral infection. Endogenous *bcl-2* overexpression was induced after a low oxidative insult of H_2O_2 in mice primary lung fibroblasts and L929 cell, whereas *bcl-2* exogenous overexpression was performed using a retroviral infection in L929 cells. Telomerase activity was quantified in *Bcl-2* overexpressing cells by the TRAP assay. When the cells were treated with different H_2O_2 concentrations, only those exposed to 50 μM showed increased telomerase activity. This correlates with BCL-2 expression as part of the endogenous response to mild oxidative stress. Oxidative stress generated during the toxic mechanism of chemotherapeutic drugs might induce BCL-2 increment, enhancing telomerase activity and reactivating the oncogenic process. Clinical trials should take into consideration the possibility of telomerase activation following increased BCL-2 expression when treating patients with ROS (reactive oxygen species) generation by anti-cancer drugs.

Keywords: *bcl-2*; BCL-2; chemotherapeutic drugs; oxidative stress; telomerase

1. Introduction

Telomerase, a ribonucleoprotein complex responsible for telomere maintenance, has been associated with cell immortalization and tumorigenesis (Greider, 1998; Blasco et al., 1995; Blackburn 2005). This enzyme is usually repressed in somatic cells (except for lymphocytes and self-renewal cells) that lose their proliferating capacity with telomere shortening and become senescence. However, when telomerase is overexpressed, cells can evade senescence and continue proliferating (Bodnar et al., 1998). Indeed, activated telomerase has been found in ~85% of human cancer tissues (Hiyama and Hiyama, 2004). Due to its central job in the maintenance of steady-state telomere length, it is a prime target for both positive and negative regulatory mechanisms.

The *bcl-2* (B-cell lymphoma-2) gene is the representative of a family of proteins that regulate cell death; it is an oncogene because its overexpression inhibits cell death (Adams and Cory, 2007; Youle and Strasser, 2008). Mutations or enhanced expression of certain genes that result in the activation of cell proliferation might synergize with *bcl-2* overexpression to induce immortality and malignant transformation.

Previous findings of our group have shown that mild oxidative stress treatment induces BCL-2 expression as a protective mechanism of cell survival (Luna-López et al., 2010). Its expression in response to oxidative stress is relevant because ROS (reactive oxygen species) are generated during the metabolism of several chemotherapeutic drugs (Chirino and Pedraza-Chaverri, 2009), but they are also known to induce pro-survival signalling pathways that can induce an adaptive response (Lau et al., 2008). This response plays a critical role in protecting cells against the cytotoxicity of anticancer agents, thus supporting the idea of a correlation between adaptation/resistance to oxidative stress to chemotherapeutic drugs (Landriscina et al., 2009). Since telomerase can contribute to cell survival and BCL-2 is seen as a predictive marker of resistance/sensitivity to chemotherapy in prognosis and stress resistance, to explore if BCL-2 increment in response to mild oxidative stress might enhance telomerase activity is worthwhile because it can either reactivate the oncogenic process or contribute to the induction of an adaptive response. This effect is important clinically because an increase in telomerase activity might be a collateral effect of chemotherapeutic drugs, which is currently not properly recognized.

Few reports have considered the relationship between BCL-2 and telomerase activity, and some of them are controversial (Mandal and Kumar, 1997; Ohmura et al., 2000; You-Wei et al., 2007). Hence, the aim of this study was to analyse if telomerase activity can be modified when BCL-2 is increased in response to a mild oxidative stress treatment in contrast with an exogenous *bcl-2* overexpression due to a retroviral infection.

2. Materials and methods

2.1. Cell culture

Primary cultures of lung fibroblasts were obtained from 2-month-old CD-1 mice as previously described (Königsberg et al., 2004). They were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 15% FBS (GIBCO-BRL), 1% non-essential amino acids, 100 units/ml penicillin and 100 μg/ml streptomycin (GIBCO-BRL) at 37°C at 95% air/ 5% CO_2). Cells were trypsinized at confluence and passaged. L929 mouse fibroblast line (ATCC Cat. No. CCL-1) were cultured in MEM (minimum essential medium; GIBCO-BRL)+10% FBS, and otherwise under the same conditions as the primary cultures.

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Abbreviations: PKC, protein kinase C; ROS, reactive oxygen species.

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2.2. Retroviral infection

Primary lung fibroblasts were infected as described by López-Diazguerrero et al. (2006), using pCL-GFPN-hBcl2 and pCL-GFPN vectors constructed and characterized by Cárdenas-Aguayo et al. (2003). Primary fibroblasts infected with the control expression vector pCL-GFPN were referred to as 'GFP+ cells', fibroblasts infected pCL-GFPN-hBcl2 vector 'Bcl-2+ cells', and 'control cells' were primary fibroblasts that were not infected. L929 fibroblasts were infected in the same way, but the cells were selected and cloned using 1 mg/ml geneticin (G418, GIBCO-BRL). With regard to the naming of the clones, L929 fibroblasts infected with pCL-GFPN-hBcl2 vector were called 'L929/Bcl-2', and the denomination 'L929' was used for L929 fibroblasts that were uninfected. BCL-2 overexpression in primary and L929 fibroblasts was determined by Western blotting of cell lysates as previously described (López-Diazguerrero et al., 2006) using a monoclonal α-BCL-2 antibody (Neomarker).

2.3. Bcl-2 endogenous overexpression in response to a mild oxidative insult

Bcl-2 overexpression was induced by mild oxidative stress (Luna-López et al., 2010). Briefly, 1×10^5 L929 cells were treated with 50, 100, 75 and 150 μM H_2O_2 for 9 h at 37°C and 95% air/5% CO_2. Total protein was isolated to determine BCL-2 expression. Cellular functionality and viability were also assessed to verify cellular integrity.

2.4. Telomerase activity

Telomerase activity was measured by the PCR-based TRAP (telomeric repeat amplification protocol) with the detection kit TRAPeze (Intergen Company) (Kim and Wu, 1997).

2.5. RNA isolation, reverse transcription and PCR analysis

Total RNA was extracted by the TRIzol® method and RNA integrity was confirmed by agarose gel electrophoresis. Total RNA from each sample was reverse transcribed into cDNA, using Superscript pre-amplification system (Invitrogen). PCR involved the following primers: *tert* (268 bp): forward: 5′-AGCAACCTCCAGCCTAACTT-3′ and: reverse: 5′-TCAAGGCATCTGTCACCTGT-3′; *gapdh* was a housekeeping control (293 bp): forward: 5′-GTGCTGAGTA-TGTCGTGG-3′ and: reverse: 5′-CACAGTCTTCGAGTGGCA-3′. PCR amplifications were performed as previously described (Luna-López et al., 2010). mRNA/expression was quantified using a Kodak IMAGEN GEL DOC, Kodak 3.1 software, normalized to the *gapdh* gene signal. Data were expressed as arbitrary units (A.U.).

2.6. Data analysis

Data are given as the means $\pm$S.D. for at least 3 independent experiments using different donor animals for the primary cultures, and 3 independent experiments for the L929 cells. ANOVA followed by the Tukey–Kramer was used to compare the densitometric data. $P<0.05$ was the minimum criterion of significance.

3. Results

3.1. BCL-2 overexpression and telomerase activity

Protein extracts from HeLa cells were used as a positive control and heated protein extracts as negatives. Under these conditions, no DNA fragments were observed (data not shown). Control and GFP+ cells contained no DNA fragments, suggesting that the enzyme was not active (Figure 1A). However, Bcl-2+ cells had demonstrable telomerase activity, which was mainly observed in the small fragment zone (10–20 bp) and was so high that the fragments could not be resolved by standard methodology. L929 extracts had basal telomerase activity because these cells are already immortal; however, when BCL-2 was increased and telomerase activity was enhanced.

3.2. BCL-2 increment due to exposure to different oxidative insults

BCL-2 increase after the treatment with different H_2O_2 concentrations was determined by a Western blot assay and the densitometric analyses were normalized against an actin control (Figure 2A). BCL-2 content is known to increase in response to a mild oxidative stress insult, as part of a mechanism of cell defence (Luna-López, et al., 2010). Here, expression of BCL-2 increased only significantly at 50 μM H_2O_2, which was referred to as an 'endogenous' BCL-2 augmentation.

3.3. Endogenous BCL-2 and telomerase activity

In examining telomerase activity in L929 cells treated with increasing concentrations of H_2O_2, longer fragments were obtained, and only a

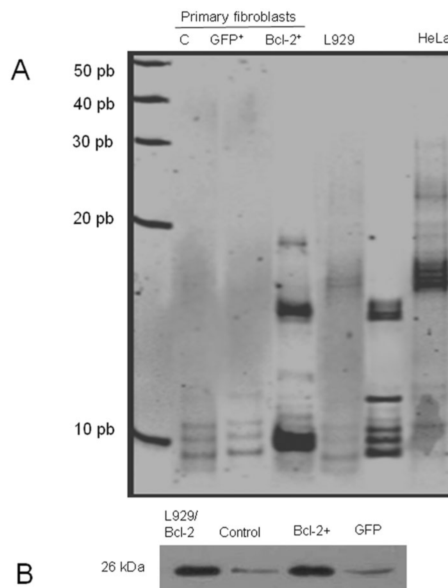

Figure 1 Telomerase activity in cells overexpressing BCL-2
(**A**) TRAP assay was used (see section 2) using protein extracts of *bcl-2* overexpressing cells and their respective controls. Primary lung fibroblasts: Control, GFP+ and Bcl-2+; L929 cell line and L929/Bcl-2. HeLa cells were used as a positive control. (**B**) Representative Western blot verifying effective BCL-2 overexpression.

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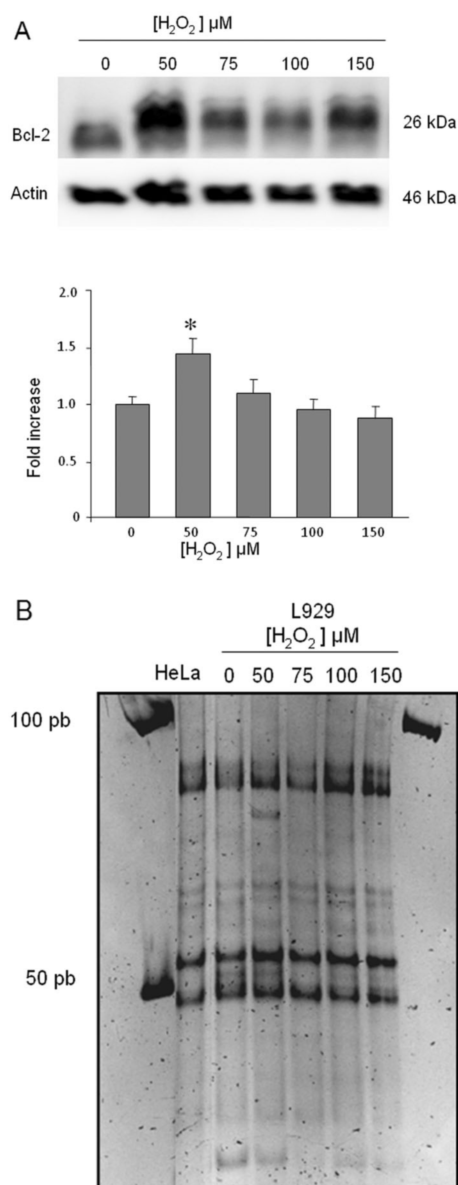

Figure 2 BCL-2 endogenous increment in response to exposure to different oxidative insults and telomerase activity

(**A**) Representative blot of protein extracts from L929 cells exposed to a range of H_2O_2 concentrations. Densitometric analysis was normalized against actin. Each point represents means $\pm$ S.D. of 3 independent experiments. Statistical significance with respect to control cells (*$P<0.05$). (**B**) Representative gel of 3 independent TRAP assays done as described in section 2 using protein extracts from H_2O_2 treated cells.

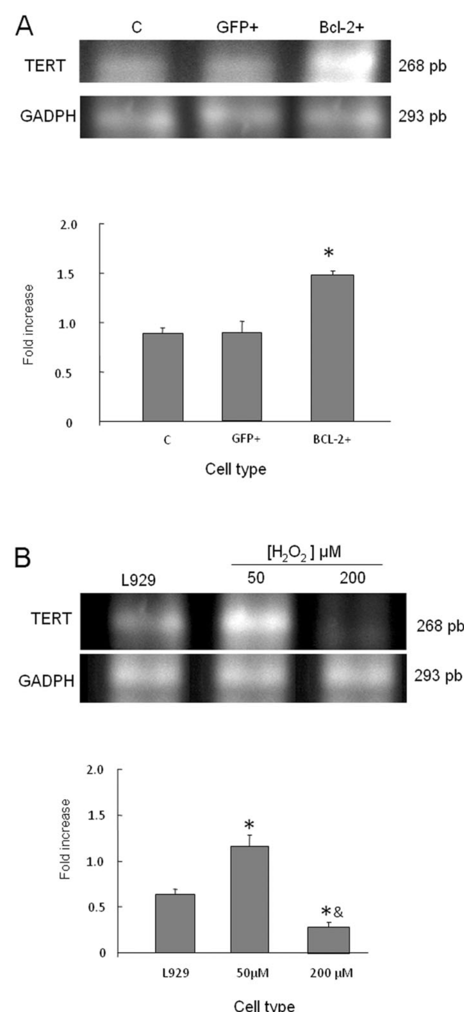

Figure 3 BCL-2 increases TERT expression

(**A**) TERT-mRNA was determined in control, GFP+ and Bcl-2+ cells as described in section 2. (**B**) TERT-mRNA in L929 treated with a mild (50 μM H_2O_2) or a severe (200 μM H_2O_2) oxidative insult to induce a *bcl-2* endogenous overexpression. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control and for densitometric analysis. Each point represents means $\pm$ S.D. of 3 determinations performed as independent experiments. *$P<0.05$ versus controls; &$P<0.05$ versus 50 μM H_2O_2 treated cells.

slight increase in telomerase activity was seen at 50 μM H_2O_2 where BCL-2 endogenously increased (Figure 2B).

3.4. BCL-2 overexpression and telomerase expression

To examine any correlation between BCL-2 overexpression and telomerase activity, TERT-mRNA was measured. When BCL-2 levels increased in Bcl-2+ cells, TERT-mRNA was also significantly increased (Figure 3A), an augmentation not observed in GFP+ and control cells. Figure 3(B) shows experiments in which L929 cells were treated with a mild H_2O_2 concentration known to induce *bcl-2* endogenous overexpression and also with a more severe oxidative insult (200 μM) that causes cellular damage (Luna-López, et al., 2010). L929 cells overexpressing BCL-2 also showed a significant increase in telomerase expression.

4. Discussion

BCL-2 can be considered a survival protein because it protects cells against oxidative stress and inhibits apoptotic cell death (Hockenbery et al., 1993; Reed, 2006). High levels of BCL-2 prevent apoptosis induced by chemotherapeutic drugs (Vargas-Roig et al., 2008; Renouf et al., 2009); therefore it is used as

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a marker of resistance and sensitivity to chemotherapy. However, since BCL-2 does not induce cellular proliferation *per se*, it is controversial whether it is of predictive potential in prognosis (Espíndola and Corleta, 2008; Vargas-Roig et al., 2008). BCL-2 might modulate steady-state ROS levels (Steinman, 1995; Susnow et al., 2009) by increasing the antioxidant protection system, particularly GSH and SOD (superoxide dismutase; Lee et al., 2001), which contribute to cell survival and resistance. Modifications of the redox state might activate different kinds of pathways and transcription factors (Forman et al., 2004; Jones, 2008). These might be general regulators that are shared by BCL-2 and telomerase. An interesting candidate is PKC (protein kinase C), which is known to induce BCL-2 expression (Weinreb et al., 2004), which might be regulated by changes in redox state. In addition, PKCα plays a pivotal role in controlling telomerase activity in human breast cancer (Li et al., 1998), whereas PKCδ is related to hTERT gene repression (Katakura et al., 2009).

Evidently in cancer progression, BCL-2's survival and protective properties encompass a physiological disadvantage related to the preservation of damaged cells that would otherwise have died. However, for tumour promotion to proceed efficiently in a cell that is protected from apoptosis due to BCL-2 overexpression, a second mutation in another oncogene is required (Youle and Strasser, 2008). Bcl-2 increase has been correlated with telomerase activation, and both are related to immortalization and tumorigenesis (Blasco et al., 1995). We found that BCL-2 expression increases as part of a survival response to a mild oxidative insult; since numerous anti-cancer drugs generate ROS as part of their toxicity, it was worthwhile determining whether BCL-2 augmentation in this response might enhance telomerase activity. If so, BCL-2 could contribute to the adaptation/resistance process or might reactivate oncogenic progression.

When the cells were treated with H_2O_2, only those exposed to 50 μM showed increased in telomerase activation, which correlates with BCL-2 expression as part of the endogenous response to mild oxidative stress. Enzyme activity was lower in endogenous overexpression compared with retroviral infection of *bcl-2* cDNA, probably because the increase in the protein levels in the endogenous *bcl-2* overexpression model are much lower than in the exogenous overexpression model. However, the important fact is that a moderate exposition to oxidative stress is sufficient to induce BCL-2 expression, which in turn stimulates telomerase activity. BCL-2 overexpression in these models also correlates with increased telomerase expression, thereby confirming our previous data. It would be of interest to see if telomerase activity continues to increase under 50 μM H_2O_2 treatment.

Physicians should take into consideration the possibility of telomerase activation along with BCL-2 increment when treating patients with ROS generating anti-cancer drugs. However, it is important to note that human and mouse telomerases differ in both their functional properties and their regulation. It is known that there is a detectable telomerase activity in normal mice cells, such that murine cells spontaneously immortalize in culture, whereas human cells rarely do so (Blasco, 2005). Therefore, the regulation of the telomerase might be different or less strict in mice than humans. For this reason, even though the experiments on murine cells might give some clues about human cancer, the results should be taken with caution in any extrapolation.

Author contribution

Norma López-Diazguerrero, Gloria Pérez-Figueroa and Cintia Martínez-Garduño isolated the primary fibroblasts, performed the retroviral infection to overexpress Bcl-2 in the primaries and determined telomerase activity in those cells. Adriana Alarcón-Aguilar cultured the L929 cell line, induced Bcl-2 overexpression and determined telomerase activity in L929. Armando Luna-López, performed the RNA isolation, reverse transcription and PCR analysis. María Gutiérrez-Ruiz analysed and discussed the data. Mina Konigsberg analysed and discussed the data, wrote the paper.

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