ORIGINAL ARTICLE

IL-2 induces apoptosis, increases the production and expression of cytochrome-C and inhibits COX IV in cervical cancer cells

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ABSTRACT

It is known that cervical cancer cells express IL-2 receptor (IL-2R) and those high doses induce cell death. To identify the type of cell death two cervical cancer cell lines, CALO and INBL, were cultured with 100 IU/ml of IL-2. Our results showed the presence of apoptotic cell death by the significant expression of phosphatidylserine on the external surface of cellular membranes, the presence of a typical DNA fragmentation and the activation of caspase 3. We also observed that the expression of COX I, COX II and COX III was not significantly altered while that of COX IV was completely inhibited. An increased expression of cytochrome-C by confocal microscopy was observed. Finally, we speculate that the clinical effect and toxicities of IL-2 used in cancer therapies is mostly due to its apoptotic effect on the cancer cells themselves rather than, as thought, the cytotoxic contribution of leukocytes.

Key Words: IL-2, Cervical cancer, Apoptosis, Cytochrome-C, Mitochondrial, Respiratory chain

1. INTRODUCTION

IL-2 is an well-known T cell growth factor that can induce cytotoxic cells capable of killing tumor cells.[1] Several clinical protocols have been developed to either administrate IL-2 in vivo or to prime cytotoxic lymphocytes in vitro, and then transfer them into the patients.[2,3] We have demonstrated that cancer cells express IL-2R[4] and that IL-2 at low doses induced their proliferation while at high doses their death.[4,5] In consequence IL-2 not only induces lymphocytes to develop toxicity against tumor cells, but it can also kill them by itself. While it is known that cytotoxic lymphocytes induce cancer cells into an apoptotic death,[6,7] it is not known the mechanism by which IL-2 kills tumor cells bearing IL-2R. In this work we evaluate if apoptosis is induced by IL-2 in cervical cancer cells (CeCa) CALO and INBL bearing IL-2R by determining the presence of phosphatidylserine on the external surface of cellular membranes, DNA fragmentation and the activation of caspase 3.[8,9] Apoptosis is known to be mediated by cytochrome-C release from mitochondria into the cytosol and with alterations in the function of the COX molecules of the mitochondrial respiratory chain.[10,11] Several authors have demonstrated that in inflammation, sepsis and several pathologies where cell death by apoptosis is present COXIV is negatively regulated.[11–17] In consequence we also evaluated changes in cytochrome-C, COXI, COXII, COXIII and COXIV expression induced by IL-2 in

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CeCa cells to evaluate whether the induction of apoptosis by IL-2 is due to an augmentation of released cytochrome-C and a negative regulation of COXIV.

2. MATERIALS AND METHODS

2.1 Reagents

Fetal bovine serum (FBS) from Hyclone Lab (Logan, UT, USA), and RPMI-1640 medium from Microlab (Mexico). Human recombinant IL-2 from R&D Systems (Minneapolis, MN, USA), annexin, active caspase 3 antibody, V/FIT-C, FITC secondary antibody and salts from Sigma–Aldrich (St Louis, MO, USA). Antibody against cytochrome-c from Santa Cruz Biotechnology (Santa Cruz, Cal, USA). All reagents for molecular biology from Promega (Wisconsin, USA). DNAzol®, TRIzol® Reagent, RNase-free DNase, reverse transcriptase and agarose from Life Technologies (New Jersey, USA).

2.2 Cell lines

CALO and INBL cell lines were established in our laboratory from two cervical carcinomas and kept in culture for 15 years. The cells were subcultured in RPMI 1640 medium with 10% FBS at 37°C with 5% CO_2.[18]

2.3 Flow cytometry for annexin and caspase 3

A flow cytometry technique was used to evaluate apoptotic CALO and INBL cells after stimulation with IL-2. In brief: Petri dishes were seeded with 5×10^5 cells and stimulated with 100 IU/ml of IL-2 during 1, 2, 3 and 4 days for annexin and 24 hrs for caspase 3. The cells were detached and washed twice with PBS by centrifugation. For the annexin evaluation the cell button was kept at room temperature in darkness for 15 minutes with 200 μl of annexin-FITC (1:200) then washed and incubated for 15 minutes with 2 μl of propidium iodide (PI) (50 μg/μl). For caspase 3 the cells were kept in darkness for 15 minutes with anti-active caspase 3 antibody and washed with PBS and analyzed in a flow cytometer (FACS Aria, BD, California, USA).

2.4 DNA fragmentation assay

Petri dishes with 2×10^6 CALO and INBL cells were seeded with 100 IU/ml of IL-2 during 1, 4, 7 and 9 days. After incubation washed by centrifugation with PBS. DNA was obtained with DNAzol® as per the manufacturer's protocol. Briefly: 500 μl of DNAzol® was added for 15 minutes on ice, followed by 1 ml of absolute ethanol. The DNA precipitate was collected and re-suspended in RNAse-free water. One microgram of DNA was used with 5μl of loading buffer (Bromophenol Blue 6x) and gel electrophoresis was performed and analyzed in a UVP® (California, USA) transilluminator.

2.5 Reverse transcription

Total RNA from CALO and INBL was extracted with TRIzol® and treated with RNase-free DNase. Complementary DNA synthesized by using 1 μg of RNA and oligo-dTprimers with reverse transcription buffer (50 mM Tris–HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$), 10 mM DTT, 200 mM of dATP, dTTP, dCTP and dGTP and 5 U of MMLV reverse transcriptase at 37°C for 1 h.

2.6 PCR amplification

PCRs for cytochrome-C and COX molecules were obtained by using 1 μg/μl of complementary DNA. 1X reaction buffer (2 mM $MgCl_2$, 0.2 mM dNTPs mix), 1 U of Taq DNA polymerase and 15 μl of RNase-free water with 30 cycles of amplification at 95°C for 1 min, followed by 55°C for 1 min and 72°C for 1 min per cycle. The amplified PCR products separated by electrophoresis on 1.5% agarose gel with TBE buffer (Tris-Borate-EDTA). The bands stained with ethidium bromide and photographed. For an internal control β-actin was used. The primers were forward and reverse; for β-actin, COX I, COX II, COX III and COX IV (see Table 1). The primers were designed using Primer3 (v.0.4.0) software from Accesolab (México City, México).

Table 1. Primers information

Primers	Forward	Reverse	Expected amplicon size (bp)
β-actin	GGG TCA GAA GGA TTC CTA TG	GGT CTC AAA CAT GAT CTG GG	234
Cytochrome-C	TTG GCA ATC CGT CAT CAG TA	CCC GAC AGT GCC TAG AAG AG	183
COX I	GTG GTT CCA AGA TGG TTG CT	CAC ATG GGG ATG GGA ATT AG	191
COX II	GGG CCT TGG ACA AGT TGT TA	CAT GAC AAA GCA GAG GCA AA	226
COX III	CCA GCT ACC ATG TCC CAG AT	TAT GCC AGC TTC CGA CTC TT	185
COX IV	GTC ACT TGG GTT TGG CCT TA	GCA AAG CAT TAG GCA AGA GG	242

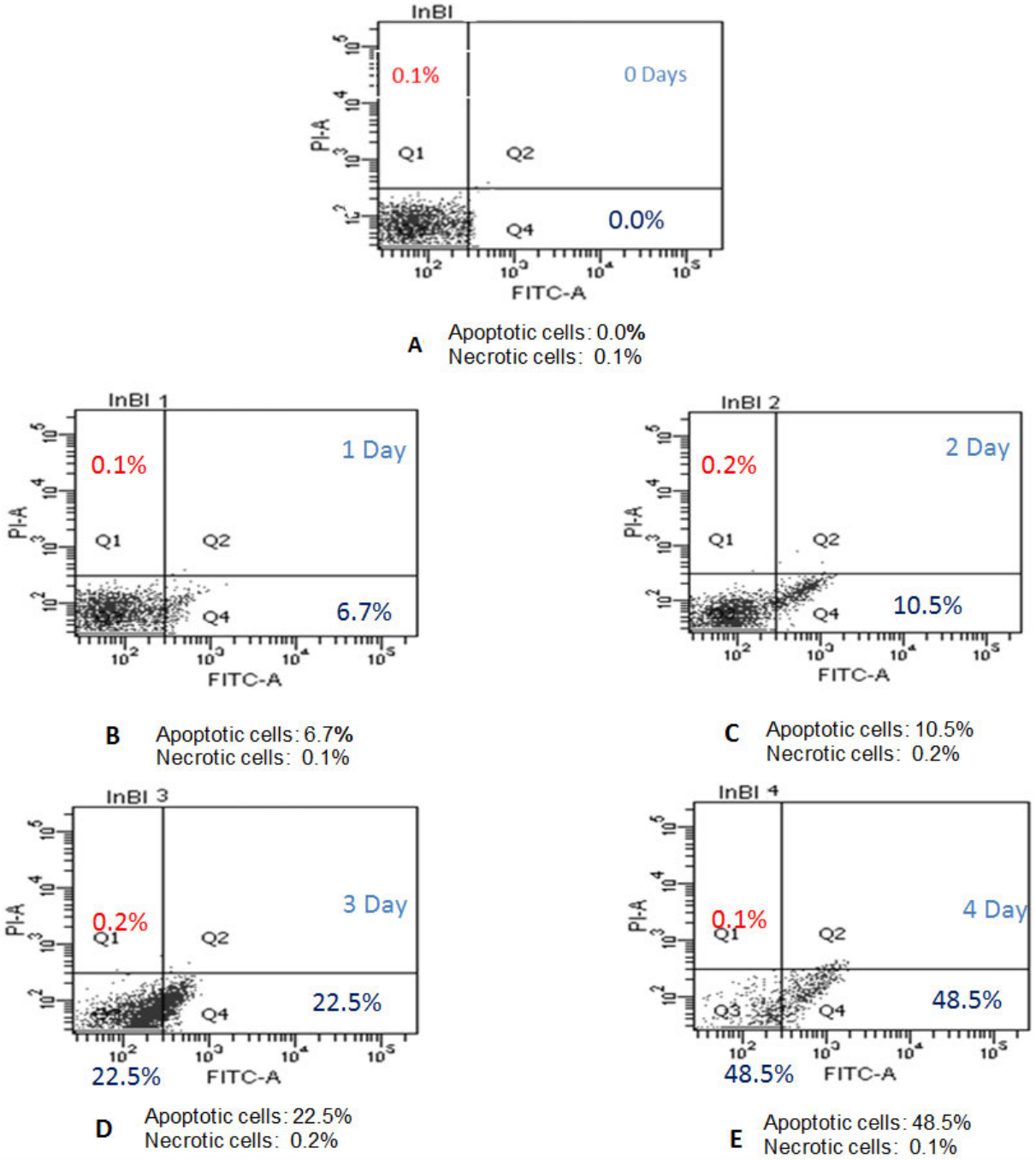

Figure 1. Determination of expression of phosphatidylserine in INBL cells with IL-2 by cytometry. (A) 0 day, (B) 1 day, (C) 2 days, (D) 3 days and (E) 4 days of culture. Q1: IP-labeled (necrotic) cells, Q2: double positive fluorescence cells, Q3: double negative fluorescence cells, and Q4: phosphatidylserine expressing (apoptotic) cells labeled with FIT-C.

2.7 Confocal microscopy

A total of 2×10^4 cells were cultured in coverslips fixed in 1% formaldehyde and permeabilized with Triton X-1000 (2%), blocked with 5% BFS, and incubated for 60 minutes with anti-cytocrome-C/FIT-C antibody. The fluorescence images were taken with a confocal microscope.

3. Results

3.1 IL-2 induces the expression of phosphatidylserine in the external surface of CALO and INBL cellular membranes

To evaluate phosphatidylserine (PI) in the cell membranes of CALO and INBL cell lines, 5×10^5 cells were cultured for 0, 1, 2, 3 and 4 days with 100 IU/ml of IL-2. Increase in

positive phosphatidylserine cells in a time depending manner was observed by cytometry (see Figures 1 and 2). By day 4 almost 50% of the cells presented this molecule on their external cell membranes. Our results also show very low necrosis, less than 3%, as determined by PI positive cells. We detected double positive cells only in CALO hinting to the presence of cells in late apoptosis for this cell line.

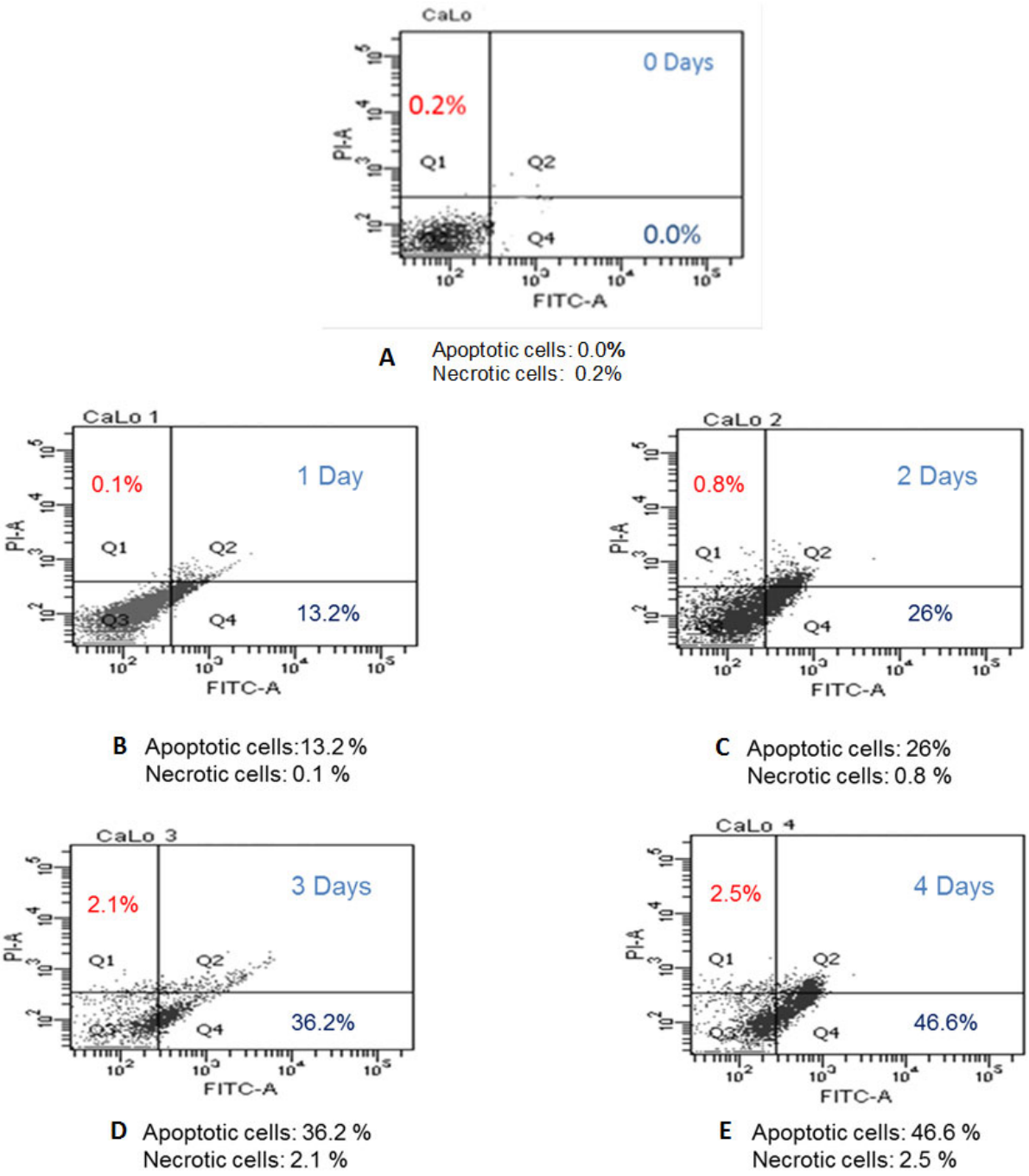

Figure 2. Determination of expression of phosphatidylserine in CALO cells with IL-2 as a by cytometry. (A) 0 day, (B) 1 day, (C) 2 days, (D) 3 days and (E) 4 days of culture. Q1: IP-labeled (necrotic) cells, Q2: double positive fluorescence cells, Q3: double negative fluorescence cells, and Q4: phosphatidylserine expressing (apoptotic) cells labeled with FIT-C.

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3.2 IL-2 induces DNA fragmentation on CALO and INBL cells

Once the presence of phosphatidylserine in the external cell membranes was induced by IL-2 was detected the possible existence of DNA fragmentation was evaluated. For this purpose, 2×10^6 cells with and without 100 IU/ml of IL-2 were cultured for 1, 4, 7 and 9 days. Our results show that only the cells with IL-2 presented a typical apoptotic fragmentation of DNA that increases in a time depending manner (see Figures 3A and 3B).

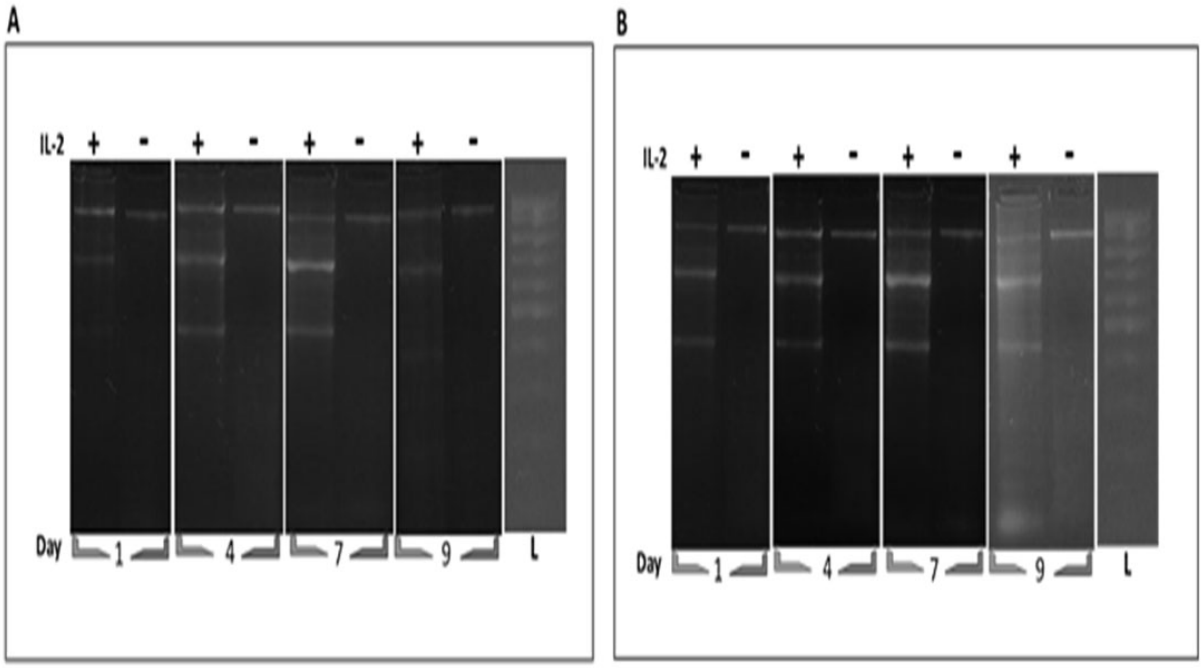

Figure 3. Determination of DNA fragmentation induced by IL-2 in CALO (A) and INBL (B) cells in a time depending manner. Cells were cultured for 1, 4, 7 and 9 days with (+) and without (-) IL-2. L: Molecular Markers.

3.3 IL-2 induces the expression of an active caspase 3 on CALO and INBL cells

Once phosphatidylserine and DNA fragmentation were evaluated, the apoptosis was further confirmed by the presence of an active caspase 3. For that purpose, 2×10^6 CALO and INBL cells were cultured for 30 min with and without 100 IU/ml of IL-2. Camptothecine was as a positive apoptotic control. Apoptosis was confirmed by significant activation of caspase 3 (see Figures 4A and 4B).

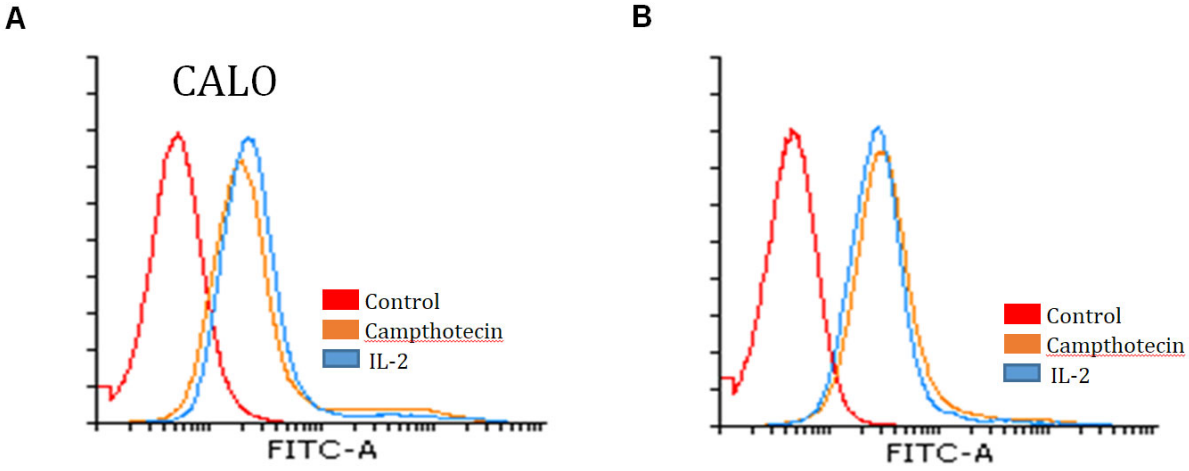

Figure 4. Presence of active caspase 3 in CALO (A) and INBL (B) cells cultured for 30 min with IL-2.

3.4 IL-2 induces cytochrome-C in CALO and INBL

A total of 1×10^6 CALO and INBL cells were cultured with 100 IU/ml of IL-2 and without (control) for 30 min, 2 h, 1 and 2 days and cytochrome-C evaluated by RT-PCR. Our results showed that after 30 min IL-2 significantly increased cytochrome-C and remained high as a function of time in culture (see Figures 5 and 6).

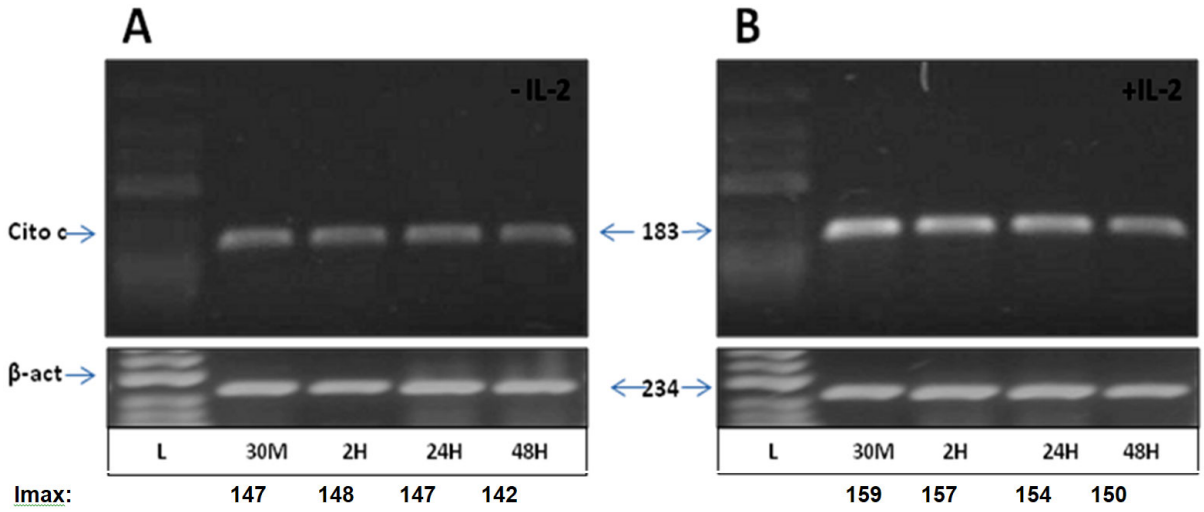

Figure 5. Amplification products for cytochrome-C (183 bp) in the CALO cell line. A) Cells cultured in the absence of IL-2; B) Cells cultured in the presence of IL-2 (which show a significant increase in the expression of cytochrome-C). β-actin (234 bp) was used as a positive control. (M: Minutes, H: Hours and L: Molecular Markers).

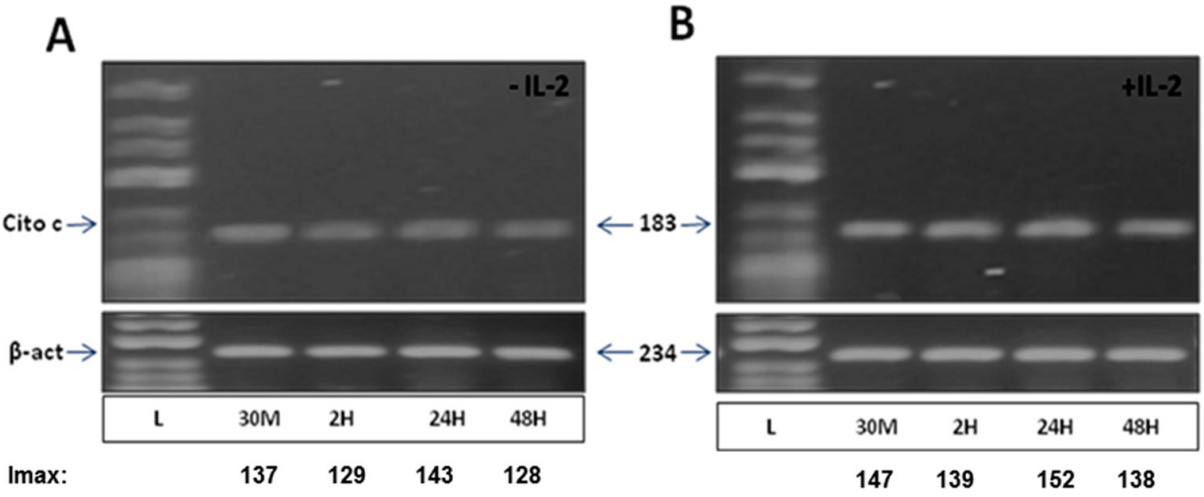

Figure 6. Amplification products for cytochrome-C (183 bp) in the INBL cell line. A) Cells cultured in the absence of IL-2. B) Cells cultured in the presence of IL- 2 (which show a significant increase in the expression of cytochrome-C). β-actin (234 bp) was used as a positive control. (M: Minutes, H: Hours and L: Molecular Marker).

3.5 Cytochrome-C is augmented in CALO and INBL by IL-2

We evaluated cytochrome-C protein expression in CALO and INBL and it was also increased. For this purpose, 2×10^4 cells were cultured with and without 100 IU/ml of IL-2 for 24 h. The cells fixed and stained with anti-cytochrome-C/FIT-C and visualized by a confocal microscope. Our results show a very strong increase in fluorescence in the cells cultured with IL-2 hinting to a significant cytochrome-C production (see Figures 7 and 8).

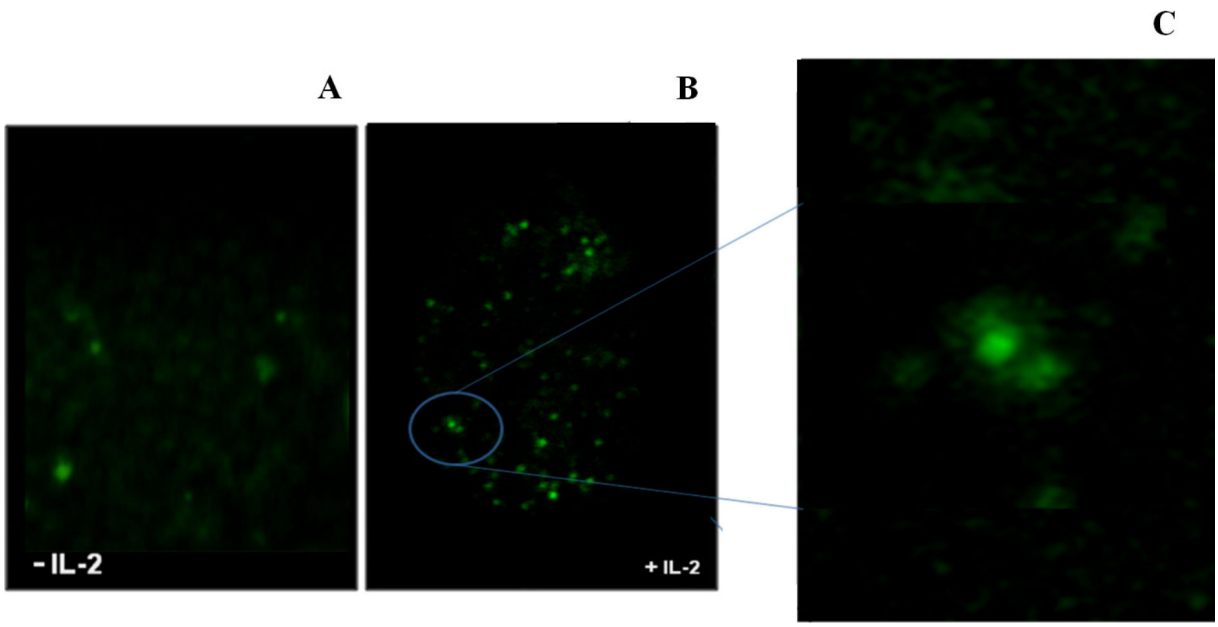

Figure 7. Confocal microscopy of FIT-C labeled anti-cytochrome-C in INBL cells cultured with IL-2 for 4 days and without IL-2 (control). A) Micrograph (40×) of cells cultured without IL-2. B) Micrograph (40×) with IL-2. C) Micrograph amplification.

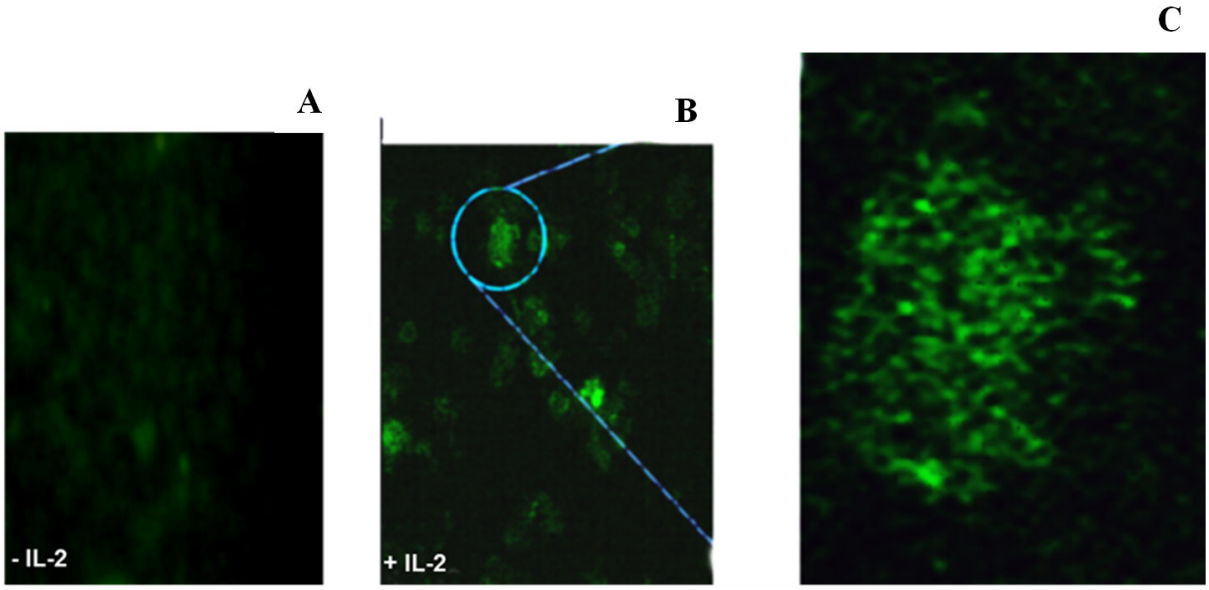

Figure 8. Confocal microscopy of FIT-C labeled anti-cytochrome-C in CALO cells cultured with IL-2 for 4 days and without IL-2 (control). A) Micrograph (40×) of cells cultured without IL-2. B) Micrograph (40×) with IL-2. C) Micrograph amplification.

3.6 IL-2 does not alter COX I, COX II and COX III on CALO and INBL while COX IV is completely inhibited

We proceeded to evaluate if there were any changes in the expression of the molecular complexes belonging to the mitochondrial respiratory chain. For this purpose, 1×10^6 CALO and INBL cells were cultured with 100 IU/ml of IL-2 and without (control) for 30 min, 2 h, 1 and 2 days and the expression of COX I, COX II, COX III and COX IV evaluated by RT-PCR. The expression of COX I, COX II and COX III remained fairly constant as a function of time (see Figures 9-14) while that of COX IV was completely inhibited (see

Figures 15 and 16). Taking into consideration that COX III reduces cytochrome-C while COX IV oxidizes, it our results point that in the apoptotic induction by IL-2 cytochrome-C is accumulated in its reduced form.

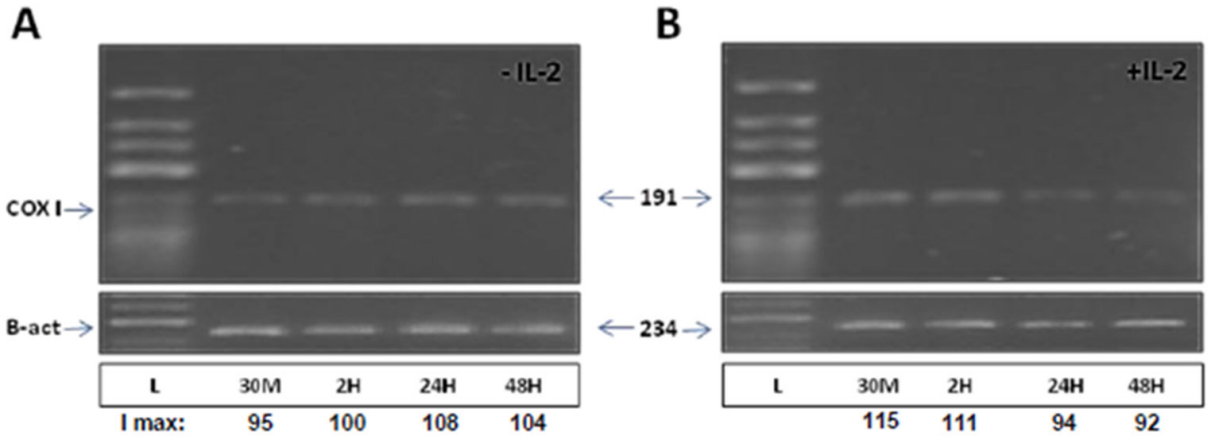

Figure 9. Amplification products for COX I-FeS (191 bp) in CALO cell line. A) Without IL-2. B) With IL-2. β-actin (234 bp) was used as a positive control. (M: Minutes, H: Hours and L: Molecular Markers). Imax: Maximum florescence.

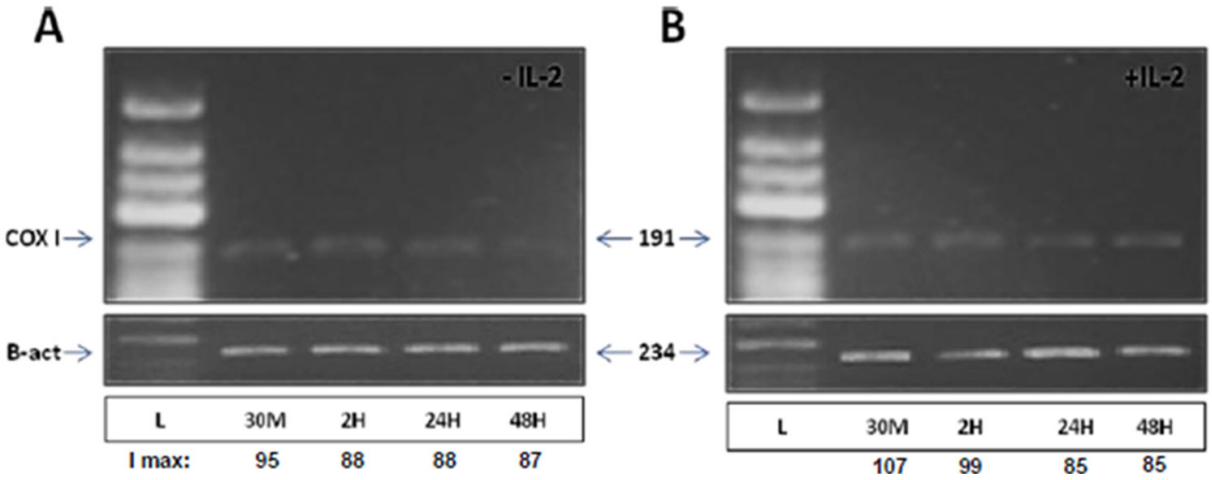

Figure 10. Amplification products for COX I-FeS (191 bp) in INBL cell line. A) Without IL-2, B) With IL-2. β-actin (234 bp) was used as a positive control. (M: Minutes, H: Hours and L: Molecular Markers). Imax: Maximum florescence.

4. Discussion

This study present evidence that, high doses of IL-2 have been administrated against cancer in order to activate cytotoxic leukocytes to kill tumor cells. The severe secondary toxicities have strongly limited its treatment. The lymphocytes under the influence of IL-2 not only became cytotoxic but also in turn secreted high amounts of other interleukins that were responsible for several of the damaging side effects.[19] Protocols were designed to deliver of IL-2 in situ to avoid the high systemic toxicity involved;[20,21] had little success due to the absolute need of a persistent high dose to maintain the anticancer effect. We recently demonstrated that cancer cells can express, as lymphocytes, a functional IL-2 receptor and that IL-2 at high doses kills them.[4,5] In consequence part of the success observed in the anticancer treatment with high doses of IL-2 was not only due to cytotoxic cells but also to its direct induction of cell death in those cells. In this respect melanoma and kidney cancer tumors whose cells express IL-2R happen to be the best responders to IL-2 treatment.[22–25]

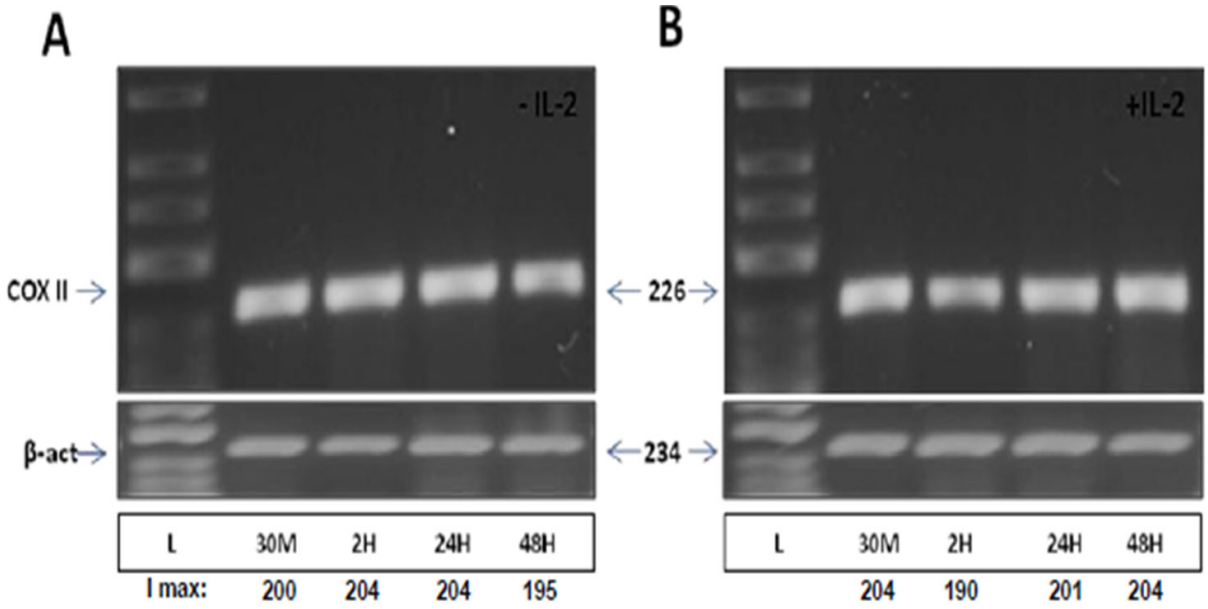

Figure 11. Amplification products for COX II-FeS (226 bp) in CALO cell line. A) Without IL-2. B) With IL-2. β-actin (234 bp) was used as a positive control. (M: Minutes, H: Hours and L: Molecular Markers). Imax: Maximum florescence.

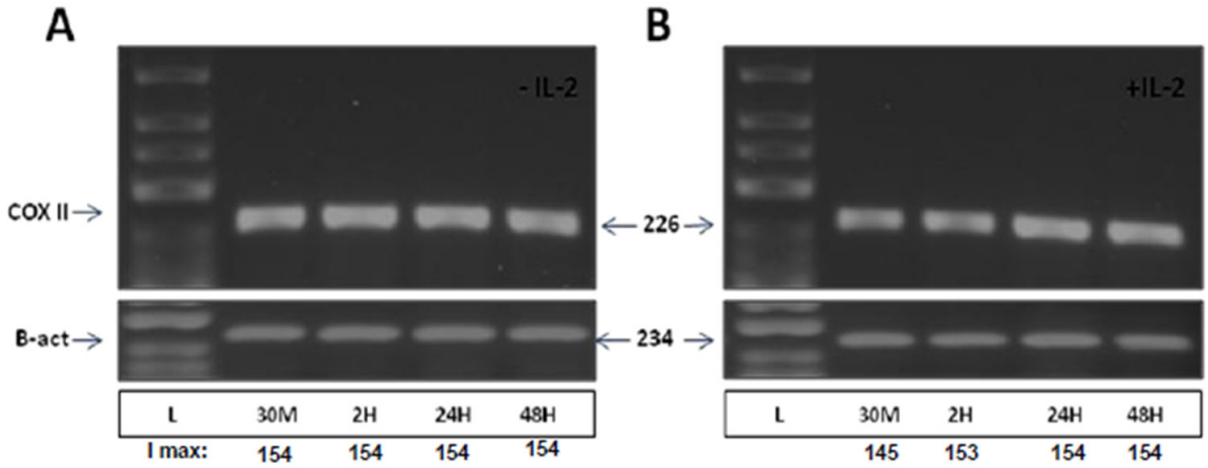

Figure 12. Amplification products for COX II-FeS (226 bp) in INBL cell line. A) Wihtout IL-2. B) With IL-2. β-actin (234 bp) was used as a positive control. (M: Minutes, H: Hours and L: Molecular Markers). Imax: Maximum florescence.

In this work we show that the mechanism by which IL-2 kills tumor cells bearing IL-2R is by apoptosis. We obtained that close to 50% of the cells presented phosphatidylserine in their external membranes while that less than 3% presented necrosis together with a typical apoptotic DNA fragmentation and the expression of active caspase 3. If IL-2 can kill IL-2R expressing cancer cells a change in strategy is needed through the use of specific vectors designed to deliver IL-2 directly to the tumor cells without the need to activate lymphocytes. For this purpose a liposome expressing IL-2 that is not covalently bound to its surface was designed and found to induce cell death in vitro and in vivo without the secondary cytotoxic effects associated when this factor was freely administrated.[26,27] Cell death by apoptosis as against necrosis has the advantage to avoid inflammation that can further complicate anticancer treatment.

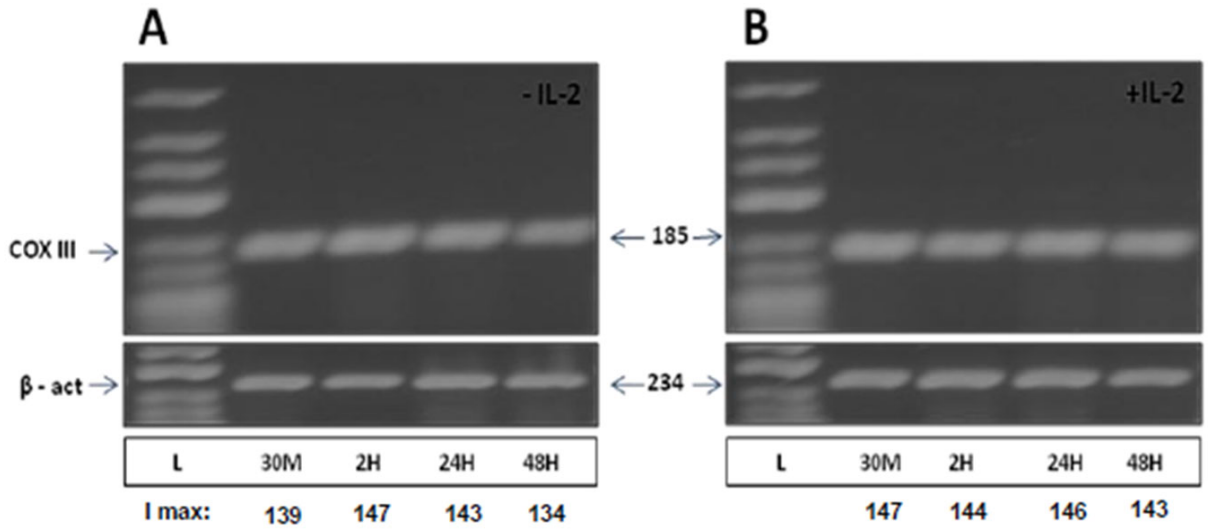

Figure 13. Amplification products for COX III-FeS (185 bp) in CALO cell line. A) Without IL-2. B) With IL-2. β-actin (234 bp) was used as a positive control. (M: Minutes, H: Hours and L: Molecular Markers). Imax: Maximum florescence.

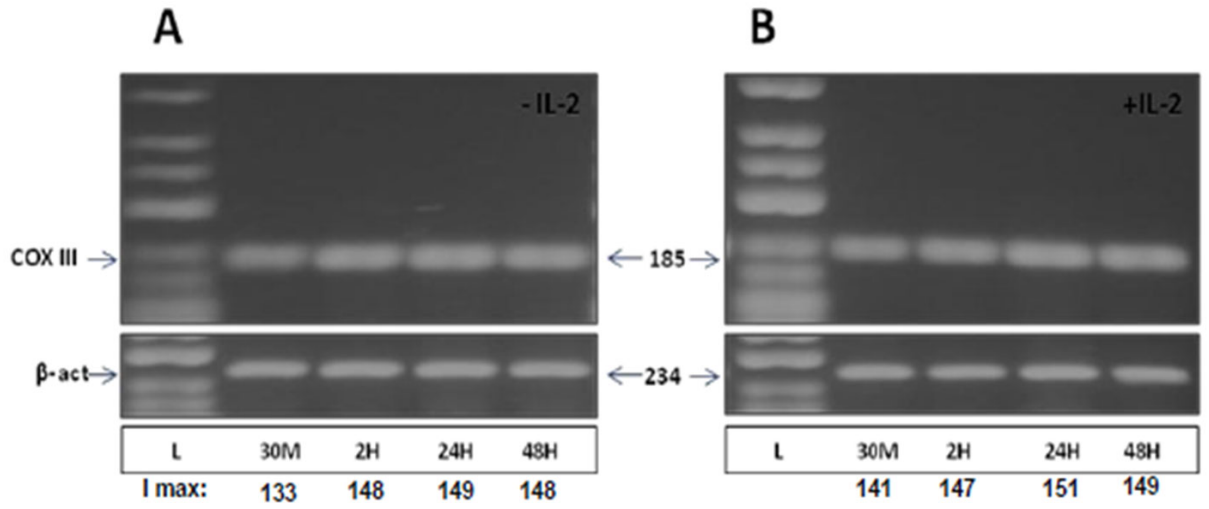

Figure 14. Amplification products for COX III-FeS (185 bp) in INBL cell line. A) Without IL-2, B) With IL-2. β-actin (234 bp) was used as a positive control. (M: Minutes, H: Hours and L: Molecular Markers). Imax: Maximum florescence.

In several pathologies it has been found a negative regulation of COXIV in their apoptotic cells.[11–17,28,29] We can thus speculate that inhibition of COXIV obtained in this work by IL-2 is the mechanism by which this molecule initiates cell death. It would be interesting to evaluate the mechanisms by which this inhibition takes place and its possible clinical applications. Because COXIV, the molecular compound responsible to oxidize cytochrome-C, was completely inhibited by IL-2, while that of COXIII responsible for its reduction remained active the accumulation of its reduced form inside the mitochondria and its probable release in this state into the cytoplasm to induce apoptosis is expected. There is a controversy whether cytochrome-C has either to be secreted in its oxidized form or in its reduced one,[10,29] or even that both are equally effective,[30] in this respect in this work we provide evidence that the secretion of its reduced form is capable to induce apoptosis.

Our results opens a new strategy to treat tumors whose cells express IL-2R by inducing apoptosis with IL-2 without the need to activate the immunological system and thus avoiding a high systemic toxicity. Epithelial cells, endothelial cells, neurons, and hepatocytes have been shown to express IL-2R[31] in consequence it would be interesting to evaluate whether their tumor cells continue to express this receptor and its susceptibility to be treated by this methodology.

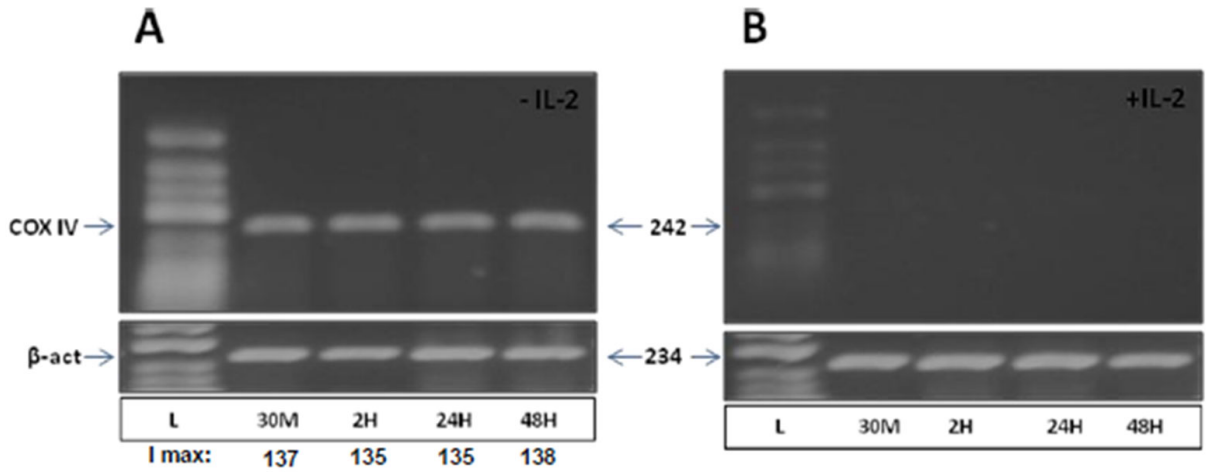

Figure 15. Amplification products for COX IV-FeS (242 bp) in CALO cell line. A) Without IL-2, B) With IL-2. β-actin(234 bp) was used as a positive control. (M: Minutes, H: Hours and L: Molecular Markers). Imax: Maximum florescence.

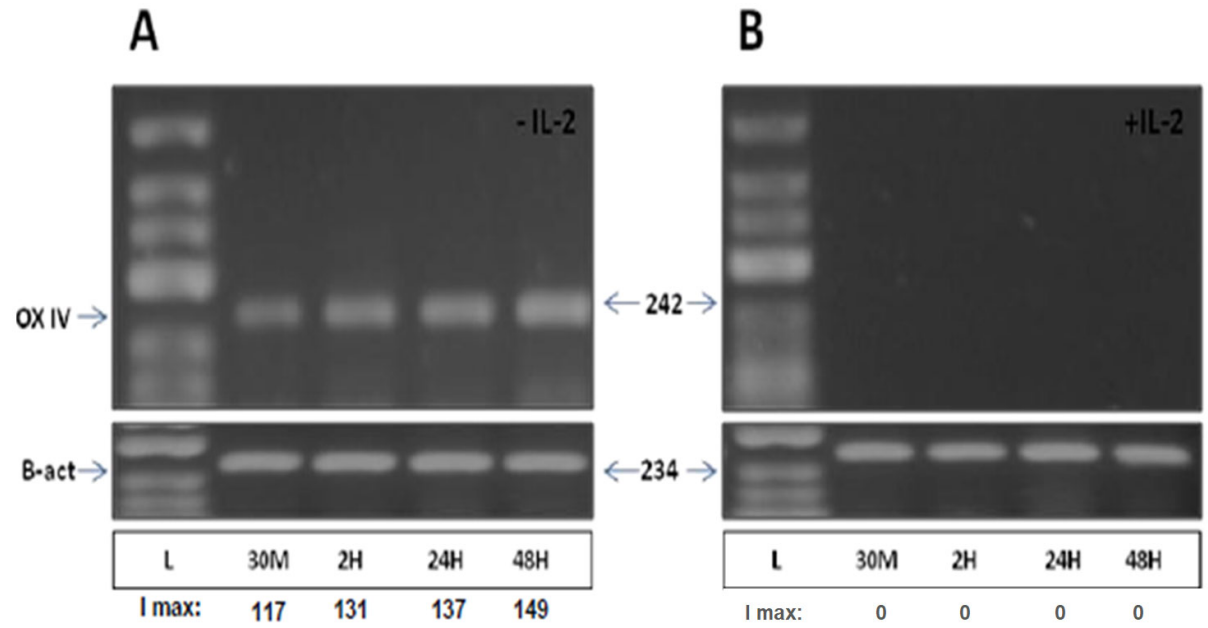

Figure 16. Amplification products for COX IV-FeS (242 bp) in INBL cell line. A) Without IL-2. B) With IL-2. β-actin (234) bp was used as a positive control. (M: Minutes, H: Hours and L: Molecular Markers). Imax: Maximum florescence.

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