The novel immunomodulator IMMUNEPOTENT CRP combined with chemotherapy agent increased the rate of immunogenic cell death and prevented melanoma growth

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Abstract. Immunogenic cell death is a cell death modality that stimulates the immune system to combat cancer cells. IMMUNEPOTENT CRP (ICRP) is a mixture of substances of low molecular weight obtained from bovine spleens that exhibits *in vitro* cytotoxic activity on different tumor cell lines and modulates the immune response *in vivo*. The aim of the present study was to determine whether the cytotoxic effect of ICRP and its combination with oxaliplatin (OXP) on murine melanoma B16F10 cells was due to immunogenic cell death. The cytotoxic assay was performed using flow cytometry to detect Annexin V and propidium iodide staining, and calreticulin (CRT) exposure. Adenosine triphosphate, heat shock protein (HSP) 70, HSP90 and high mobility group box 1 (HMGB1) release were identified using bioluminescence, western blot and ELISA assays, respectively. The present *in vitro* study demonstrated that treatments with ICRP or OXP induced cell death in a time-dependent manner, but treatment with the combination of ICRP + OXP increased the cytotoxic effect following 24 h of treatment. CRT exposure and release of adenosine triphosphate (ATP), HSP70, HSP90 and HMGB1 were induced by treatment with ICRP, and the combination of ICRP + OXP increased the exposure and release of damage-associated molecular patterns (DAMPs), while OXP treatment only induced CRT exposure, ATP and HMGB1 release. The *in vivo* experiments demonstrated that administration of tumor-derived DAMP-rich cell lysates derived from B16F10 cells treated with ICRP and the combination of ICRP + OXP prevented melanoma growth; however, OXP treatment did not. These results suggested that IMMUNEPOTENT CRP may be used as an agent to increase the ability of antitumor drugs to induce immunogenic cell death and prevent the growth of melanoma.

Introduction

Immunogenic death is a cell death modality that stimulates the innate and adaptive immune system against cell death associated antigens, inducing tumor cell immunogenicity. Immunogenic cell death (ICD) is characterized by the exposure or release of immunogenic molecules by dying tumor cells (1-3), termed damage-associated molecular patterns (DAMPs). Certain DAMPs include calreticulin (CRT) exposure on the outer surface of the plasma membrane, which serves an important function as a phagocytic signal, stimulating phagocytes to engulf dead tumor cells (3,4-8). The secretion of adenosine triphosphate (ATP) by dying tumor cells is an important chemoattractant for macrophages and dendritic cells to the site of tumor (3,9-13). The heat shock proteins (HSP) 70 and HSP90, and non-histone chromatin binding protein high mobility group box 1 (HMGB1) are released into the extracellular space and promote the recognition of tumor cells by dendritic cells by binding to receptors on the cell surface, leading to their elimination by the immune system (14-19).

It has been suggested that only certain types of cancer therapies induce immunogenic cell death *in vitro* (20-23) and *in vivo* (5,24-29), and that these may be classified into two groups. The targets of group I ICD inducers include DNA

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and repair machinery proteins, cytosolic proteins, plasma membrane or nucleic proteins, which are targeted by chemotherapeutic agents including anthracyclines, oxaliplatin (OXP) and mitoxantrone; cardiac glycosides, shikonin and ultraviolet C irradiation. Group II ICD inducers target the endoplasmic reticulum, and include photodynamic therapy with hypericin and Coxsackievirus B3 (8,30-34). Certain ICD agents with these characteristics are considered to be anti-cancer vaccines, and as therapies that prevent residual cancer.

IMMUNEPOTENT CRP (ICRP) is a dialysate of a heterogeneous mixture of low-molecular-weight substances released from the disintegrated leukocytes of the blood or lymphoid tissue obtained from homogenized bovine spleens. ICRP exhibits *in vitro* cytotoxic effects on different tumor cell lines and modulates the immune response *in vivo* (35-40). The aim of the present study was to determine whether ICRP or ICRP combined with OXP induced ICD and prevented melanoma growth.

Materials and methods

Reagents and antibodies. OXP was obtained from Teva Pharmaceutical Industries, Ltd. (Petah Tikva, Israel). IMMUNEPOTENT CRP was produced by the Department of Immunology and Virology, Biological Sciences Faculty, Autonomous University of Nuevo Leon (Nuevo Leon, Mexico). Propidium iodide staining solution and allophycocyanin (APC)-conjugated Annexin V was obtained from BD Pharmingen (BD Biosciences, San Jose, CA, USA). Phycoerythin (PE)-conjugated CRT monoclonal antibodies (cat. no. ADI-SPA-601PE-F) and IgG1 isotype control monoclonal antibodies (cat. no. ADI-SAB-600PE-D) were obtained from Enzo Life Sciences (Farmingdale, NY, USA). Mouse monoclonal antibodies targeting HSP70 (cat. no. sc-24), HMGB1 (cat. no. sc-56698), β-actin (cat. no. sc-69879), rabbit polyclonal IgG antibody targeting HSP90 α/β (cat. no. sc-7947), and secondary antibodies including mouse anti-rabbit (cat. no. sc-2357) and goat anti-mouse (cat. no. sc-2005) IgGs conjugated to horseradish peroxidase were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Complete Halt Protease inhibitor cocktail (100X) was obtained from Thermo Fisher Scientific, Inc. (cat. no. 87786; Waltham, MA, USA). The ENLITEN ATP Assay System Bioluminescence Detection kit for ATP measurement was obtained from Promega Corporation (Madison, WI, USA). The HMGB1 BioAssay ELISA kit (mouse; cat. no. 194487) was purchased from US Biological Life Sciences (Salem, MA, USA).

Cell line and culture conditions. The murine melanoma B16F10 cell line was obtained from American Type Tissue Collection (Manassas, VA, USA) and was maintained in Dulbecco's modified Eagle's medium/F-12 medium 1:1 containing 2.50 mM L-Glutamine, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer medium (cat. no. SH30023.FS; all HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (cat. no. 10082147) and 100 U/ml penicillin/streptomycin (cat. no. 15140122; both Gibco; Thermo Fisher Scientific, Inc.). The cell line was incubated in a humidified atmosphere with 5% CO_2 at 37°C.

Cell death assays. B16F10 cells ($1x10^5$) were seeded into 12-well plates and cultured overnight in 5% CO_2 at 37°C. Cells were treated with ICRP (1 U/ml), OXP (800 μM) or a combination of ICRP (1 U/ml) + OXP (800 μM) for 24, 48 and 72 h. Following treatment, cells were collected and washed with phosphate-buffered saline (PBS) and resuspended in 100 μl of 1X binding buffer (0.1 M Hepes pH 7.4, 1.4 M NaCl and 25 mM $CaCl_2$; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with APC-conjugated Annexin V (5 μl/sample) and propidium iodide (1 μl/sample), incubated on ice and kept in the dark for 15 min. Flow cytometry analysis was performed using an Accuri C6 cytometer; BD Accuri C6 Software version 1.0.264.21 was used for data analysis (both BD Biosciences, San Jose, CA, USA).

Analysis of CRT on the cell surface. Flow cytometry was used to determine the level of CRT exposure induced by the treatments. B16F10 cells ($1x10^5$) were treated with ICRP (1 U/ml), OXP (800 μM) or the combination of ICRP (1 U/ml) + OXP (800 μM) for 6, 12, 24, 48 or 72 h. The cells were harvested, suspended in 1X PBS with 1% fetal bovine serum and incubated for 1 h at room temperature in the dark with a CRT monoclonal antibody (dilution, 1:100), then analyzed.

Western blot analysis. B16F10 cells ($5x10^6$) cells were treated with ICRP (1 U/ml), OXP (800 μM) or a combination of ICRP (1 U/ml) + OXP (800 μM) for 24, 48 and 72 h. Following this, supernatants and cells were collected and centrifuged at 260 x g for 10 min at room temperature. The cells were washed with PBS and homogenized using the SET 2X lysis buffer (20 mM Tris pH 6.8, 2 mM EDTA pH 8.0, 300 mM NaCl and 4% SDS; Sigma-Aldrich; Merck KGaA) supplemented with the Complete Halt protease inhibitor cocktail. Protein quantification was performed using a detergent compatible Lowry protocol protein assay (cat. no. 5000112; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal amounts of soluble proteins (50 μg) were resolved by 12% SDS-PAGE and transferred to a nitrocellulose membrane. Non-specific binding sites were blocked by incubating the membrane for 1 h at room temperature in TBS-Tween-20 supplemented with 5% non-fat powdered milk followed by overnight incubation at 4°C with primary antibodies, including anti-HSP90 or -β-actin (the internal standard) at a dilution of 1:500, or anti-HSP70 or -HMGB1 at a dilution of 1:400. The antibodies were diluted in 10 ml of 1X TBS-0.1% Tween-20 buffer supplemented with 5% w/v BSA. Primary antibodies were detected by incubation with mouse anti-rabbit or goat anti-mouse IgGs conjugated to horseradish peroxidase, diluted to 1:2,000, for 2 h at room temperature. The protein bands were visualized using an enhanced chemiluminescence western blotting detection kit and high performance chemiluminescence film (both GE Healthcare Life Sciences).

ATP release assays. Extracellular ATP levels were measured in the supernatant of B16F10 cells treated with ICRP (1 U/ml), OXP (800 μM) or a combination of ICRP (1 U/ml) + OXP (800 μM) for 24, 48 and 72 h by luciferin-based ENLITEN ATP assay (Promega Corporation) according to the manufacturer's protocol. Chemiluminescence was recorded with a
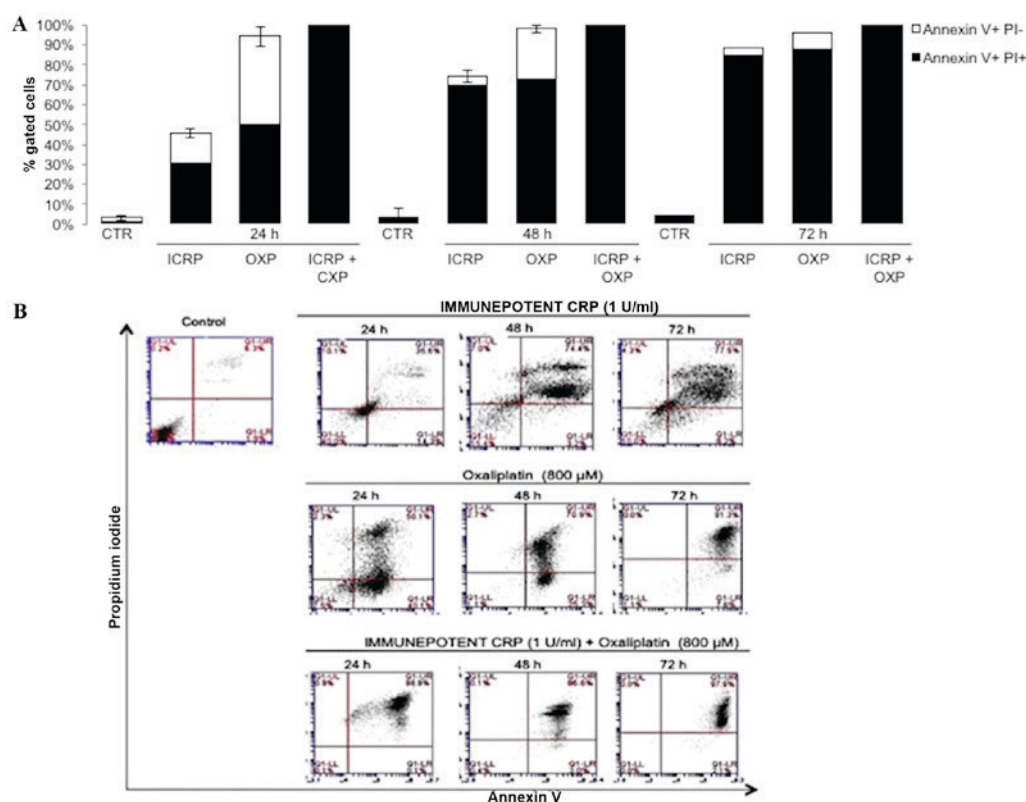
Figure 1. ICRP, OXP and ICRP + OXP treatments induce cell death in B16F10 cells, as assessed by Annexin V-allophycocyanin and PI staining and analyzed by flow cytometry. (A) Representative bar graphs of data obtained via flow cytometry, presented as the mean ± standard deviation and performed in triplicate. (B) Data are represented as a dot plot. ICRP, IMMUNEPOTENT CRP; OXP, oxaliplatin; PI, propidium iodide; CTR, control.

BioTek Synergy microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

HMGB1 release assays. HMGB1 concentration was measured in the supernatant of untreated or treated B16F10 cells with ICRP (1 U/ml), OXP (800 μM) and the combination of ICRP (1 U/ml) + OXP (800 μM) for 24, 48 and 72 h using the HMGB1 ELISA kit according to the manufacturer's protocol.

Animals. A total of 20 female C57BL/6 mice were purchased from Harlan Laboratories (Mexico City, Mexico). The body weight of mice was 23 (±2) g and they were between 6 and 10 weeks of age. The mice were housed at 25-29°C with 45% humidity and a 12 h light: 12 h dark cycle. Mice were provided food and water *ad libitum*. According to experimental protocols that were approved by the Ethics Review Committee for Animal Experimentation of the Biological Sciences Faculty, Autonomous University of Nuevo Leon (San Nicolas de los Garza, Mexico).

In vivo anti-tumor vaccination experiments. A total of $5x10^6$ B16F10 cells were treated with ICRP (3 U/ml), OXP (12,600 μM) or a combination of ICRP (1.2 U/ml) + OXP (900 μM) for 48 h *in vitro*. Following this, the cells were centrifuged at 260 x g for 10 min and washed twice with PBS. Finally, cells were resuspended in 200 μl PBS and inoculated subcutaneously into the left flank of the mice. After 7 days, mice were challenged with $5x10^5$ live B16F10 cells resuspended in 200 μl of PBS via subcutaneous injection into the right flank. Tumor incidence and growth were measured every day at the

two injection sites for 60 days with a digital caliper. Tumor volume was calculated using the formula: V=(W (2) x L)/2, where V is tumor volume, W is tumor width and L is tumor length.

Humane end-points were used to avoid unnecessary suffering. Mice were sacrificed when the width of tumors reached 20 mm. Effort was made to minimize environmental stress. The control group was sacrificed at 20 days, the OXP group at 30 days, and the ICRP and ICRP + OXP groups at 60 days.

Statistical analysis. The experiments were performed in triplicate and statistical analysis was performed using a one-way analysis of variance followed by Dunnett's test. P<0.05 was considered to indicate a statistically significant difference. SPSS version 17.0 (IBM Corp., Armonk, NY, USA) was used to perform the analysis.

Results

Cell death is induced by ICRP, OXP or ICRP + OXP treatments in the B16F10 cell line. Treatment with ICRP [24 h (30.6%), 48 h (69.6%), and 72 h (85%)] and OXP [24 h (50.5%), 48 h (72.6%), and 72 h (88%)] induced cell death in a time-dependent manner (Fig. 1A). Treatment with the combination of ICRP + OXP induced a cytotoxic effect at all time-points evaluated [24 h (98.8%), 48 h (100%), and 72 h (100%); Fig. 1A]. Apoptosis was indicated by staining with Annexin V-APC, which binds to phosphatidylserine residues on the surface of dying cells, and propidium iodide, which
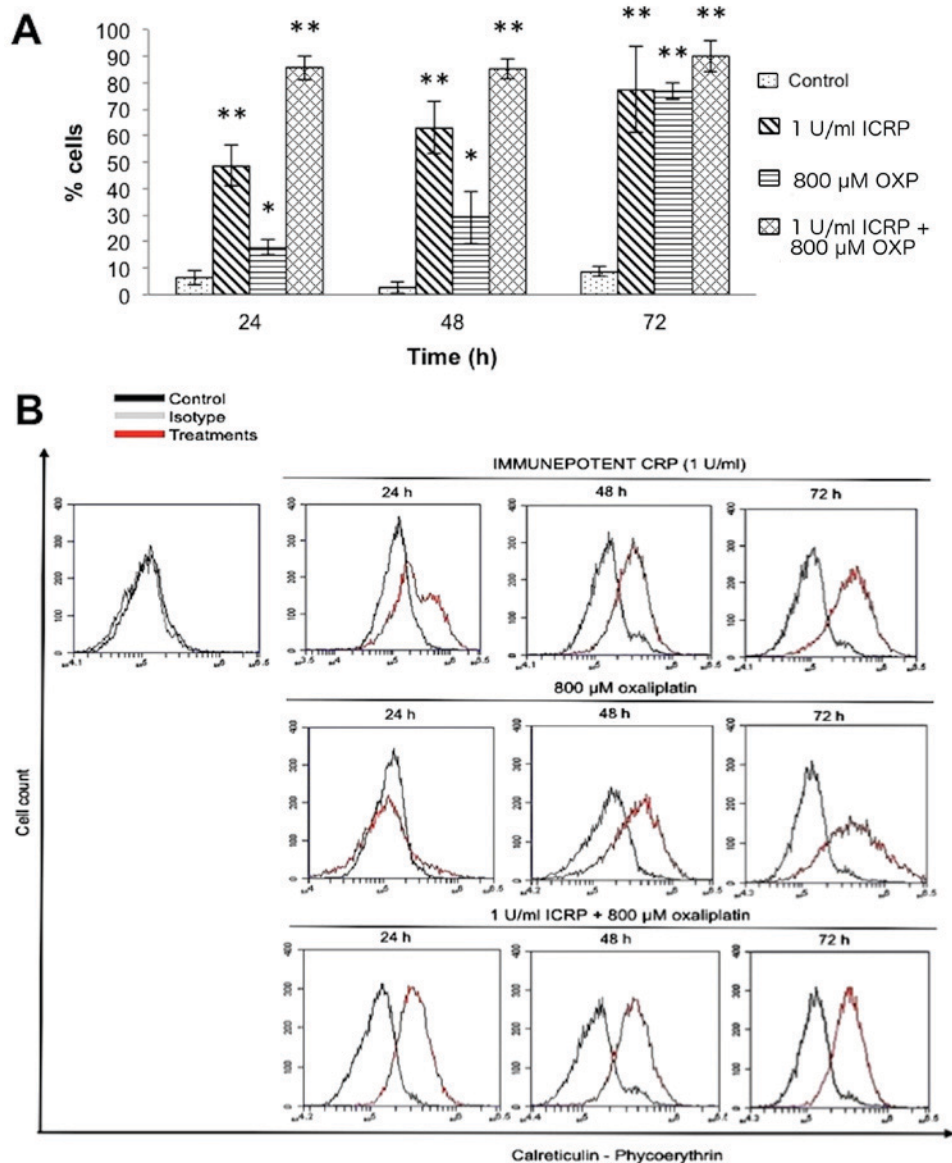
Figure 2. ICRP, OXP and ICRP + OXP treatments induce exposure of calreticulin in B16F10 melanoma cells. Treated cells were stained using anti-calreticulin antibodies and isotype control antibodies conjugated with fluorochrome phycoerythrin and analyzed by flow cytometry. Results are from three independent experiments performed in triplicate, and data are presented as the mean ± standard deviation. (A) Representative bar graphs of data obtained from flow cytometry. *P<0.05, **P<0.001 vs. control. (B) Representative dot plots of the experiment. ICRP, IMMUNEPOTENT CRP; OXP, oxaliplatin.

penetrates only into dead cells (Fig. 1B). In the untreated B16F10 cells, cell viability was not affected.

CRT exposure is induced by treatment with ICRP, OXP or ICRP + OXP in the B16F10 cell line. Treatment with ICRP or OXP induced CRT exposure in a time-dependent manner (ICRP: 6 h, 9.1%; 12 h, 23%; 24 h, 48.6%; 48 h, 63%; and 72 h, 77.4%; P<0.001; OXP: 6 h, 2.6%; 12 h, 9.2%; 24 h, 17.7%; 48 h, 29%; and 72 h, 76.8%; P<0.05; Fig. 2). Treatment with a combination of ICRP + OXP induced the highest exposure of CRT following treatment for 24 h, however, following combined treatment for 6 h, CRT exposure was higher compared with the individual treatments (6 h, 12.7%; 12 h, 58.2%; 24 h, 85.4%; 48 h, 85.10%; and 72 h, 90%; P<0.001; Fig. 2).

Release of ATP in the B16F10 cell line following treatment with ICRP, OXP or ICRP + OXP. The release of ATP

significantly increased following 24 h of treatment with ICRP (P<0.05; Fig. 3) and the combination of ICRP+OXP (P<0.001; Fig. 3). Following 48 h of treatment, ATP release was not detected except by ICRP treatment at 72 h (P<0.01; Fig. 3). The decreased ATP detection following 48 h of treatment may be because extracellular ATP is not stable, due to the presence of various enzymes that degrade ATP (ATPases) or the decomposition of ATP to adenosine diphosphate, adenosine monophosphate, adenosine and inorganic phosphate (10,13).

Release of HSP70, HSP90 and HMGB1 proteins in the B16F10 cell line treated with ICRP, OXP or ICRP+OXP. In the supernatant, treatment with ICRP + OXP induced the release of HSP70 and HSP90 in the B16F10 cells at all evaluated time-points, and their release was increased at 48 and 72 h relative to 24 h. ICRP treatment induced the release of these proteins, and they were detected at 48 h,
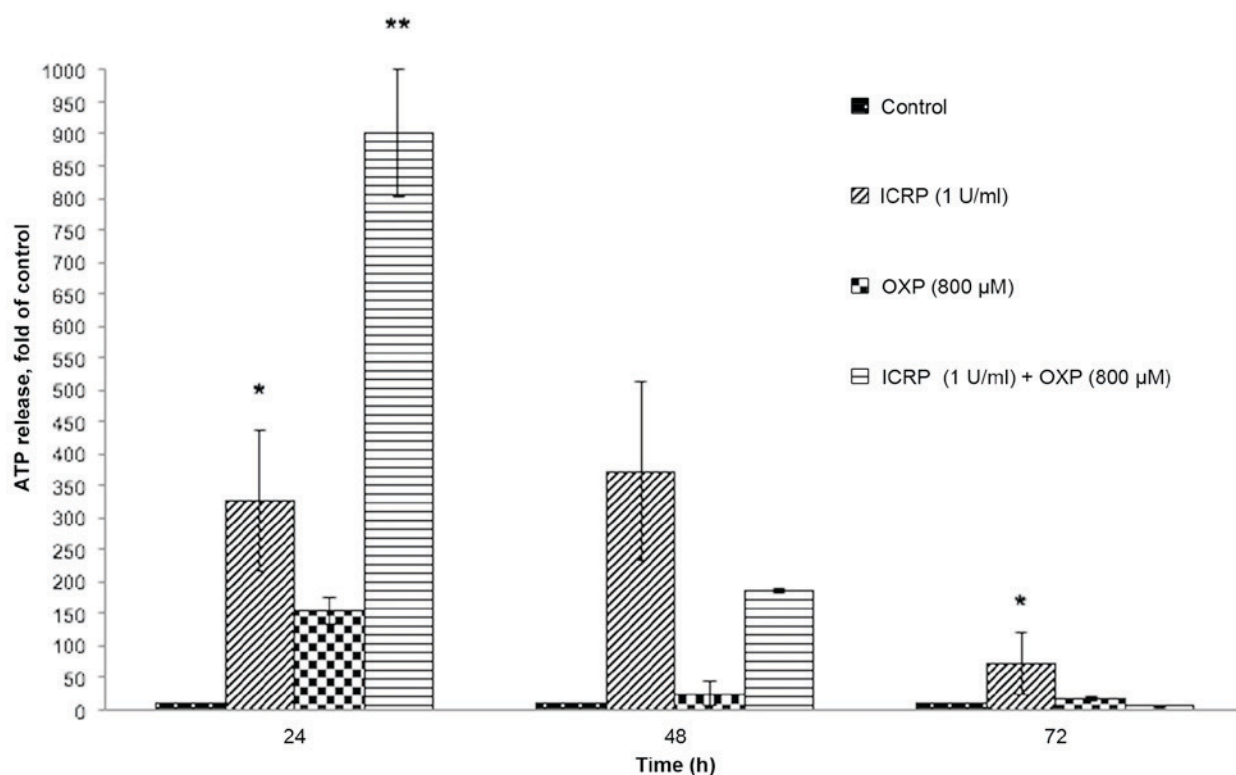
Figure 3. ATP release in response to ICRP, OXP, and ICRP + OXP treatments. [*]P<0.05, [**]P<0.001 vs. control. ATP, adenosine triphosphate; ICRP, IMMUNEPOTENT CRP; OXP, oxaliplatin.
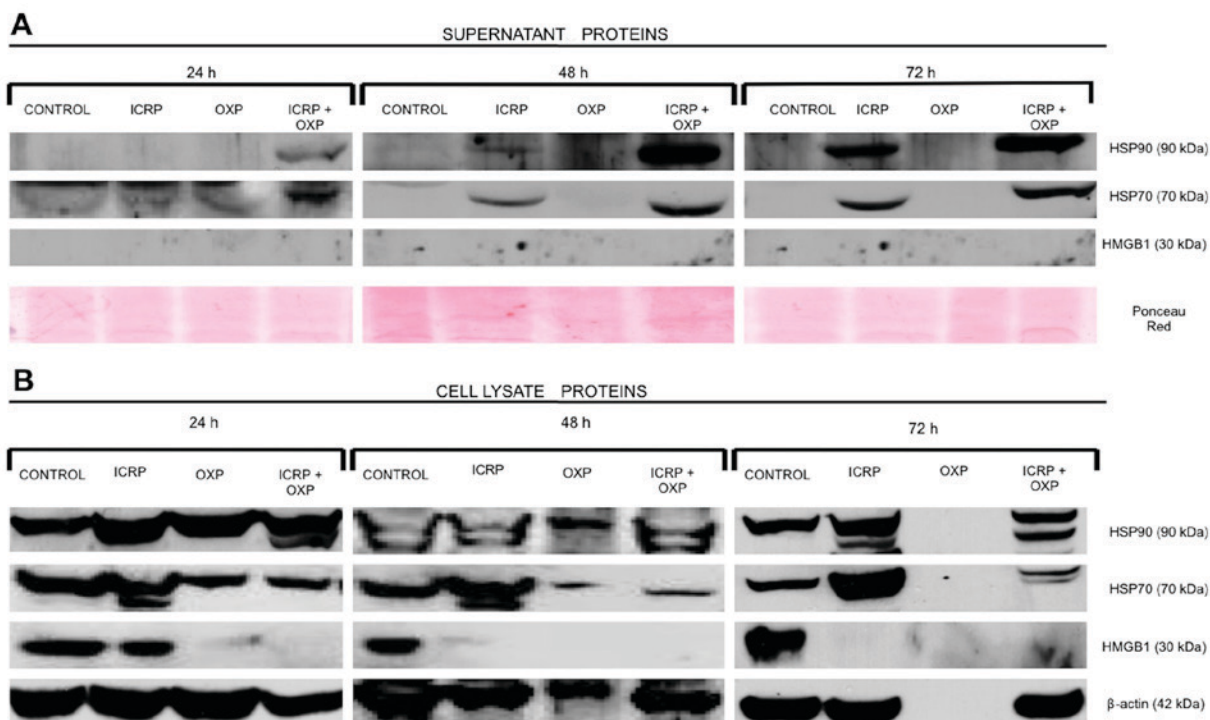

Figure 4. Expression and release of HSP70, HSP90 and HMGB1 proteins in response to treatment with ICRP, OXP, and ICRP + OXP. Western blotting was performed using (A) cell supernatants and (B) whole cell lysates of B16F10 cells. β-actin was used as a loading control. HSP, heat shock protein; HMGB1, high mobility group box 1; ICRP, IMMUNEPOTENT CRP; OXP, oxaliplatin.

and their release was increased in the supernatants at 72 h relative to 24 h. OXP did not induce the release of HSP70 or HSP90 at any of the evaluated time-points. HMGB1 was not detected in any of the treatments by western blotting (Fig. 4). Therefore, an ELISA kit with high sensitivity was used for its detection, and it was revealed that all ICRP, OXP or ICRP
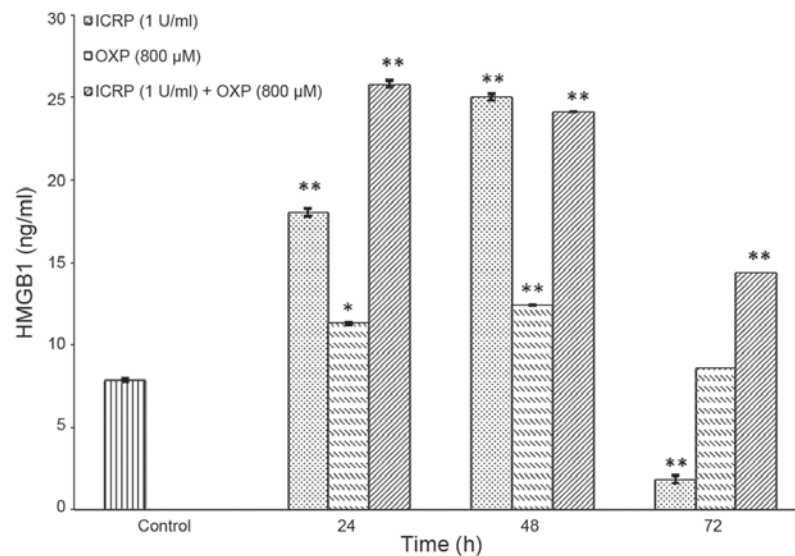
Figure 5. HMGB1 release by dying B16F10 cells following treatment with ICRP, OXP, and ICRP + OXP, as assessed by ELISA. The results represent three experiments performed in triplicate and data are expressed as the mean ± standard deviation. *P<0.05, **P<0.001 vs. control. HMGB1, high mobility group box 1; ICRP, IMMUNEPOTENT CRP; OXP, oxaliplatin.
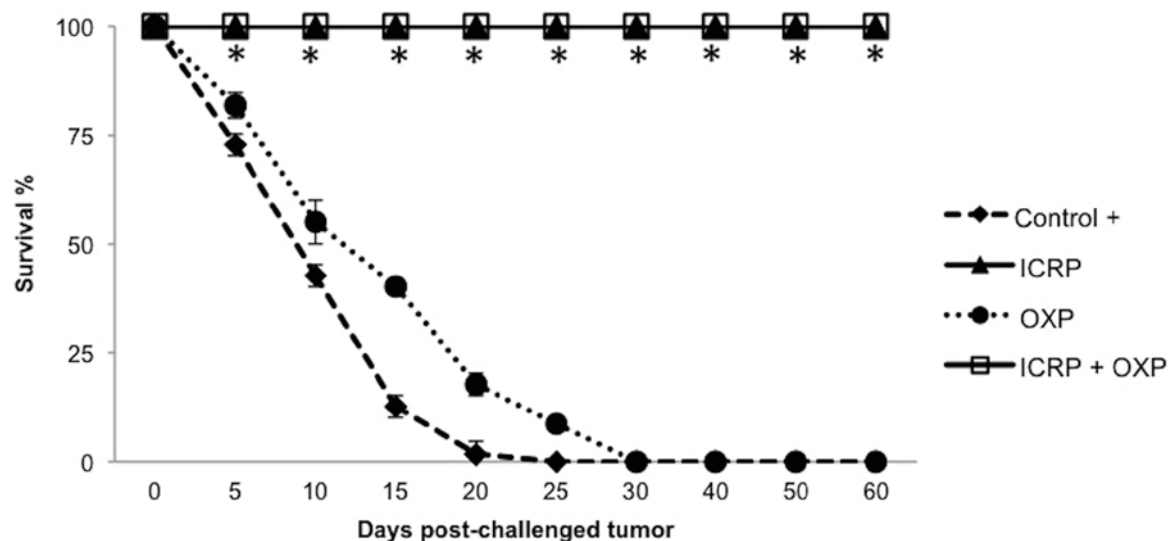

Figure 6. Effects of tumor-derived DAMP-rich cell lysates derived from B16F10 cells treated with ICRP, OXP, and ICRP + OXP in the prevention of melanoma. The tumor-derived DAMP-rich cell lysate-derived treatments of B16F10 cells were subcutaneously inoculated into C57BL/6 mice on the left flank. Mice were re-challenged with live B16F10 cells on the right flank. After 7 days, melanoma growth was evaluated. *P<0.05 vs. control. DAMP, damage-associated molecular pattern; ICRP, IMMUNEPOTENT CRP; OXP, oxaliplatin.

+ OXP treatments increased HMGB1 release at 24 and 48 h compared with the control (P<0.001; Fig. 5). Treatment with ICRP significantly increased the release of HMGB1 at 24 h (P<0.001) and 48 h (P<0.001) compared with the control; however, it decreased at 72 h (P<0.001; Fig. 5). Treatment with OXP significantly increased the release of HMGB1 at 24 h (P<0.05) and 48 h (P<0.001) but decreased the release at 72 h compared with the control (Fig. 5). The treatment with ICRP + OXP significantly increased the release of HMGB1 at all the times evaluated (P<0.001) compared with the control. In the cell lysates, the presence of HSP70, HSP90 and HMGB1 was detected in the control treatment at all the time-points evaluated. HSP70 and HSP90 were detected in cells exposed to ICRP or ICRP + OXP treatments at 24, 48

and 72 h, but were not detected in cells exposed to OXP treatment at 72 h. HMGB1 only was detected in cells exposed to ICRP treatment at 24 h (Fig. 4).

In vivo effects of tumor-derived DAMP-rich cell lysates derived from B16F10 cells treated with ICRP, OXP or ICRP + OXP in the prevention of melanoma. *In vitro* experiments demonstrated the cytotoxic effect and induction of DAMPs, which are characteristic of immunogenic cell death, following treatments with ICRP, OXP, or a combination of ICRP + OXP. To evaluate the immunogenicity of the immunogenic cell death induced by the treatments *in vivo*, anti-tumor vaccination experiments were performed in a mouse model. The tumor-derived DAMP-rich cell lysates derived from previous

treatments of B16F10 cells with ICRP, OXP or a combination of ICRP + OXP were administered to mice prior to inoculation with live B16F10 cells. The results demonstrated that the ICRP and ICRP + OXP treatments prevented the development of melanoma growth. The tumor-derived DAMP-rich cell lysates from the OXP treatment did not protect against melanoma growth, but delayed mortality in the mice (30 days) compared with the control (20 days; Fig. 6).

Discussion

There has been increasing interest in the optimization of old and the identification of novel therapeutic agents with the capacity to generate anticancer immunity. Immunogenic cell death generated from antitumor treatments is one of the mechanisms through which these treatments elicit their tumor-targeting immune responses (41). The present study aimed to determine the potential of ICRP used in combination with chemotherapy to increase cytotoxicity against tumor cells and to induce molecules associated with immunogenic death, as novel therapeutic regimens that focus on a combination of strategies to trigger these mechanisms are in development.

The results of the present study demonstrated that treatment with ICRP was cytotoxic in B16F10 melanoma cells, and that the combination of ICRP treatment with OXP increased the rate of cell death. This was similar to results concerning the cytotoxic effects on cancer cells of ICRP treatment alone, which were identified previously by our group (37). It is important to note that in the present study, the treatments with ICRP or ICRP + OXP induced the release of several immunogenic molecules (CRT, ATP, HSP70, HSP90 and HMGB1) *in vitro*. The presence of these molecules, when induced by anthracycline treatment in colon cancer CT26 cells, melanoma B16F10 cells or fibrosarcoma MCA205 cells, has been associated with the prevention of tumor growth (42-44). HMGB1 was not detected in the supernatant by western blot assay, potentially due to the sensitivity of the antibody used; but when examined with an ELISA kit with high sensitivity the release of HMGB1 was detected. Similar results for human HMBG1 were demonstrated by Nowak *et al* (45), where concentrations of 150 ng/ml were detected by ELISA and western blot, but lower concentrations (1 or 15 ng/ml) were not detected by western blot; only by ELISA.

OXP treatment has been suggested to induce the release of DAMPs (CRT, ATP, HMGB1 and type I interferon) in several cancer cell lines, and is considered to be an inductor agent of ICD (27,31). To the best of our knowledge, studies examining the effect of immunogenic cell death induced by OXP on the B16F10 cancer cell line, which is poorly immunogenic, had not yet been performed. The results of the present study indicated that oxaliplatin induced the exposure of CRT and the release of ATP and HMGB1, but did not induce the release of HSP70 and HSP90 in the B16F10 cancer cell line. Compounds with the capability to induce the release of HSP70, HSP90, ATP and HMGB1 have been demonstrated to prevent tumor growth (30-34,46,47). In addition, Chen *et al* (24) suggested that treatment with shikonin in B16F10 cells induced exposure of CRT and the release of HSP70, HSP90, GRP78 and HMGB1 *in vitro*, and allowed the maturation of dendritic cells; and shikonin tumor-derived cell lysate-loaded dendritic cell vaccines were indicated to induce retardation of tumor growth and to increase the survival rate of mice. Similar results were obtained in the present study, where tumor cell lysates derived from B16F10 cells treated with ICRP or ICRP + OXP administered to mice were demonstrated to prevent melanoma growth induced by injection with live B16F10 cells, indicating the potential of these treatments to induce immunogenic cell death. OXP-induced immunogenic death of colon cancer cells has been demonstrated in murine and human cell lines, and OXP prevented the formation of tumors (4,27). However; in the present study this was not observed, suggesting that it is necessary for OXP to induce the additional release of HSP70 and HSP90 in B16F10 cells in order to generate immunogenic cell death, similar to the aforementioned treatments (30-34,48,49). Depending on the type of cell death inducer involved, tumor cells may expose or release factors that affect their uptake (CRT), maturation (HSP90) or antigen presentation by dendritic cells (HMGB1) (46). Studies investigating the induction of immunogenic cell death by anthracyclines, OXP or ionizing radiation suggest that these effects require the presence of DAMPs and their corresponding receptors on antigen-presenting cells for complete therapeutic success (46).

It has been demonstrated that in cancer patients or whole tumor cells, treatment with drugs including oxaliplatin or doxorubicin (30-34) or physical procedures (47,50-52) may induce a specific immune response through tumor antigens that have been exposed to dendritic cells, together with DAMP priming and the activation of naïve T cells to target tumors (53).

Although additional studies are necessary to understand the mechanisms underlying the prevention of melanoma growth, the present study demonstrated that IMMUNEPOTENT CRP, currently used in Mexico as an adjuvant to the immune system, may be used in combination with chemotherapy as a potential agent to increase the action of antitumor drugs by inducing immunogenic cell death to eliminate residual cancer cells in patients, and may generate the development of a whole tumor vaccine.

Acknowledgements

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