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The Action of Bovine Cartilage on Tumor Cells *In Vitro* And *In Vivo*

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Abstract: In an earlier study we had investigated the effect of Bovine Cartilage (BC) on mouse melanoma cells. In pursuant to this study BC anti-tumor activity *in vitro* against several human tumor cell lines was evaluated, mechanism by which BC induces tumor cell death was studied and its immunogenicity was assessed. Five mice received Intraperitoneal (IP) injection of BC once every 14 days for over a period of 42 days. Fourteen days after the last BC dose, mice were bled and sera were used to assess production of anti-BC antibodies by passive hemagglutination. To assess the effect of BC on human tumor cells, three different human tumor cell lines were incubated separately with increasing concentrations of BC for 48 h and percent viability was determined *in vitro*. Moreover, human lung cancer cell line A549 and mouse B16F10 melanoma cells were incubated separately with their respective half maximal inhibitory concentration (IC50) of BC and apoptosis/necrosis assay was performed. No antibody against BC was detected. *In vitro*, total eradication of human tumor cell lines was seen with 5000 μg mL^{-1} of BC. It appears that BC induces tumor cell death through apoptosis and this mechanism of action is the same across different cell lines and species. Additionally, BC appeared to be non-immunogenic.

Keywords: Bovine Cartilage, MDA-MB-231, A549, PC3

Introduction

It has been reported that Bovine Cartilage (BC) has several useful medical properties including acceleration of wound healing, an anti- inflammatory property and an anti-tumor activity.

John F. Prudden was the first to assess the ability of Bovine Cartilage to accelerate healing in experimental wounds as well as in chronically non healing human wounds (Prudden *et al*., 1975; Takayuki, 1960; Prudden *et al*., 1962; 1964; Prudden, 1964; Prudden and Allen, 1965). Subsequently, a study conducted by Houck *et al*. (1961) showed that, in addition to having ability in wound healing, BC presents a potent anti-inflammatory activity. This anti-inflammatory ability of BC further triggered Prudden to assess its role in treating cancer (Prudden, 1985). For this purpose 31 terminally ill patients, each with a different type of malignancy, were subjected to BC treatment; Prudden registered some significant improvements in a wide variety of intractable malignancies (Prudden, 1985).

The mechanism of action underlying the anti-tumor activity of BC is not well defined. At least four possible mechanisms have been proposed; BC might directly induce cancer cell death (Prudden, 1985; Durie *et al*., 1985), it could stimulate the immune system (Rosen *et al*., 1998; Morell and Daniel, 2014), it might inhibit collagenase activity (Kuettner *et al*., 1977; Murray *et al*., 1986) and/or it could inhibit angiogenesis (Folkman *et al*., 1971; Langer *et al*., 1976).

The use of BC to accelerate wound healing and treat inflammatory diseases has been adopted by the physicians in medical fields, but its anti-tumor activity was not further investigated.

Earlier, we investigated the effect of BC on the survival of B16F10 melanoma cells and mouse mononuclear cells (Tanelian *et al*., 2016). The aim of this study was to further evaluate the *in vitro* anti-tumor activity of BC against human tumor cell lines, determine the mode by which it induces tumor cell death and assess its immunogenicity.

Materials and Methods

In Vitro Evaluation of BC Effect

BC used was "NOW Bovine Cartilage" (395 S. Glen Elyn Rd, Bloomingdale, IL), a commercially available extract. The powdered BC was weighed, dissolved in sterile distilled water and stored at -20°C until used.

Cell Lines and Culture Conditions

MDA-MB-231 breast cancer cell line, A549 human lung carcinoma cell line and PC3 human prostate adenocarcinoma cell line were generously provided by Dr. Marwan El-Sabban from the department of Anatomy, Cell Biology and Physiology at the American University of Beirut. MDA-MB-231 cells were cultured in RPMI-1640, supplemented with10% Fetal Bovine Serum (FBS) and 1% Pen-Strep, whereas A549 and PC3 cells were cultured in a medium containing RPMI-1640, 10% FBS, 1% L-Glut and 1% Pen-Strep. All cells were incubated at 37°C in a humidified incubator with 5% CO_2.

Proliferation Assays

MDA-MB-231, A549 and PC3 cells were grown at 80% confluency, trypsinized, counted and seeded in 24-well plates at a seeding density of 2×10^4 cells/500µl/well, 1.8×10^4cells/500 µl/well and 1.7×10^4 cells/500µl/well respectively. Cells were then incubated for 24 h prior to treatment by 500 µL of five different BC concentrations (5, 10, 100, 1000 and 5000 µg mL^{-1}) for 48 h. No BC was added to the control wells and the samples were run in duplicates. After 48 h of incubation the cells were collected and a viable cell count was determined with a haemocytometer using the trypan-blue exclusion assay.

Determination of Apoptosis

Apoptosis was detected in B16F10 melanoma and A549 cells using the ApoDETECT Annexin V-FITC Kit, following the manufacturer's protocol. Briefly, cells were seeded in 24-well plates with their respective growth medium at 2×10^4 cells/well and 1.8×10^4cells/well respectively. The cells were treated with their respective IC50 of BC ($IC50_{A549}$ = 189 µg mL−1and $IC50_{B16F10}$ = 167 µg mL^{-1}) for 48 h, collected by centrifugation and washed with ice cold PBS. The cells were then re-suspended in 1X binding buffer at a cell density of 2-5×10^5 cells/mL. Ten microliters of Annexin V-FITC were added to 190 µL of cell suspension and incubated for 10 min at room temperature. Finally, the cells were washed and PI was added on the re-suspended cells in 190 µL binding buffer and analyzed by flow cytometry.

In Vivo Study

Immunogenicity Assessment of Bovine Cartilage

The use of mice in this study was approved by the Institutional Animal Care and Use Committee (IACUC) at the faculty of Medicine, American University of Beirut. Ten C57BL/6 female mice, 6 to 8 weeks old, were divided in to two groups (5 mice in each). One group remained untreated, while the second group received IP injection of 75 mg mL^{-1} of BC at a dose of 0.4 ml/ mouse. The treatment was given once every 14 days for 42 days.

On day 56, 14 days after the last injection, all mice were anesthetized, dissected and blood was collected by cardiac puncture. Blood from each group was pooled and serum was separated and used for immunogenicity assessment by passive Hemagglutination using two different methods: Neter's method (Neter *et al*., 1956) and Boyden's Method (Boyden, 1951).

Statistical Analysis

Whenever applicable, data were expressed as a Mean ± SD. The IC50 of BC was calculated using GraphPad Prism 5.0. The unpaired student's T-test was performed for Statistical comparisons using Graph pad online software. Results were considered to be statistically significant at P value <0.05.

Results

In Vitro Results

Viability of Human Tumor Cells Incubated with BCs

The anti-tumor activity of BC was assessed, after exposing human tumor cells, MDA-MB-231, A549 and PC3 cells, to increasing range of BC concentrations. After 48 h of incubation, similar anti-tumor response was seen against the three human tumor cells. Significant decrease in the viable MDA-MB-231, A549 and PC3 cell count was obtained in the wells treated with 500 µL of 5 µg mL^{-1} (P value $_{MDA\text{-}MB\ 231}$ = 0.003, P value $_{A549}$ = 0.0012, P value $_{PC3}$ =0), 10 µg mL^{-1} (P value $_{MDA\text{-}MB\ 231}$ = 0.005, P value $_{A549}$ = 0.0001, P value $_{PC3}$ = 0.0121) ,100 µg mL^{-1} (P value$_{MDA\text{-}MB\ 231}$ = 0.0013, P value$_{A549}$ = 0.0014, P value $_{PC3}$ = 0.0059) and 1000 µg mL^{-1} (P value $_{MDA\text{-}MB\text{-}231}$ = 0.001, P value $_{A549}$ = 0.001, P value $_{PC3}$ = 0.0014) of BC, when compared to their respective control wells. Total eradication of MDA-MB-231, A549 and PC3 cells was obtained in the wells treated with 500 µL of 5000 µg mL^{-1} of BC (Fig. 1).

Determination of Apoptosis and Necrosis using Flow Cytometry

To determine the mode of tumor cell death, untreated and BC-treated B16f10 melanoma and A549 lung carcinoma cells were stained with annexin V and

PI. Flow cytometry analysis of stained cells can differentiate cells into four groups, namely viable (annexin V- PI-), early apoptosis (annexin V+ PI-), late apoptosis (annexin V+ PI+) and necrotic (annexin V- PI+) cells. As shown in Fig. 2 and 3, after 48 h of BC exposure, the majority of B16F10 melanoma and A549 lung carcinoma cells showed early and late apoptotic characteristics compared to their respective controls, where early apoptosis was the dominant feature in both tumor cell population. Moreover, the flow cytometry results revealed no significant difference between the percentages of necrotic cells for each BC-treated cell and their respective controls. Less than 2% of the BC-treated population showed necrotic signs.

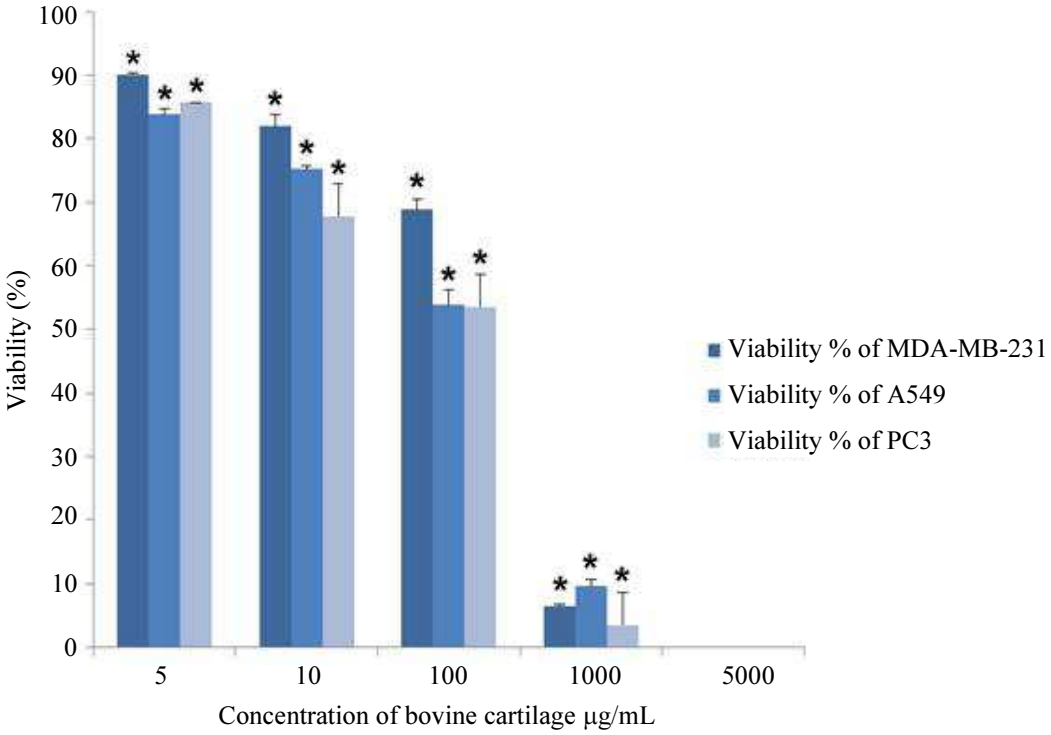
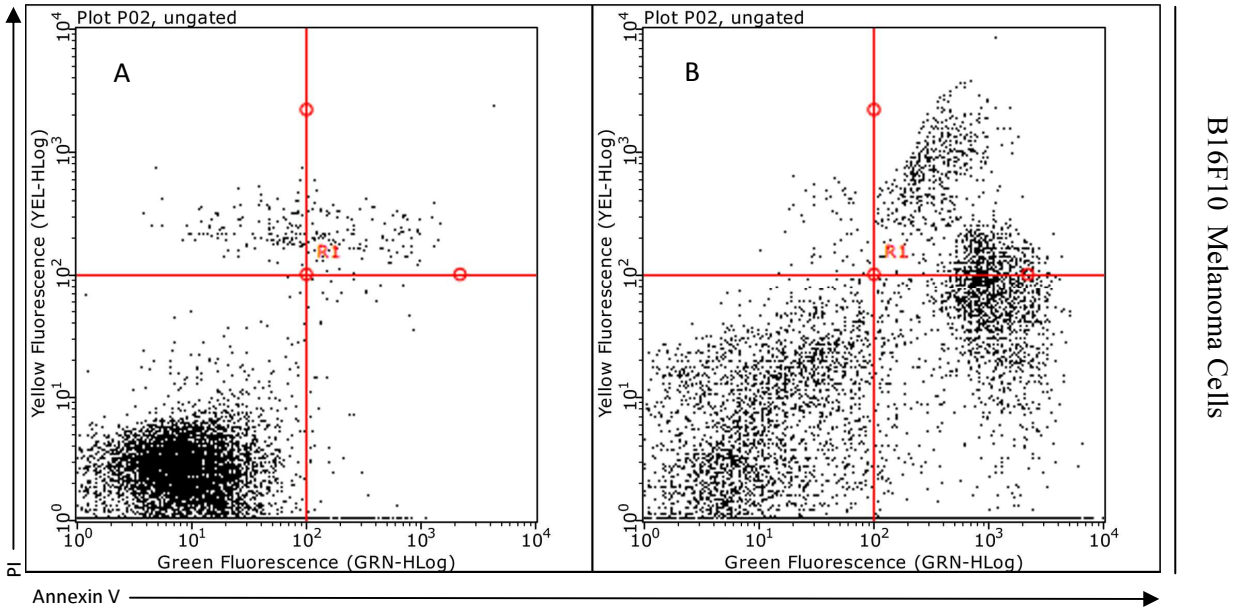


Fig. 1: Assessing the effect of BC on the growth of MDA-MB-231, A549, and PC3 cells *in vitro*. The viability of human tumor cells significantly decreased with increasing concentration of BC, reaching a total eradication of cells at 5000 µg mL*: Statistically significant at p-value <0.05



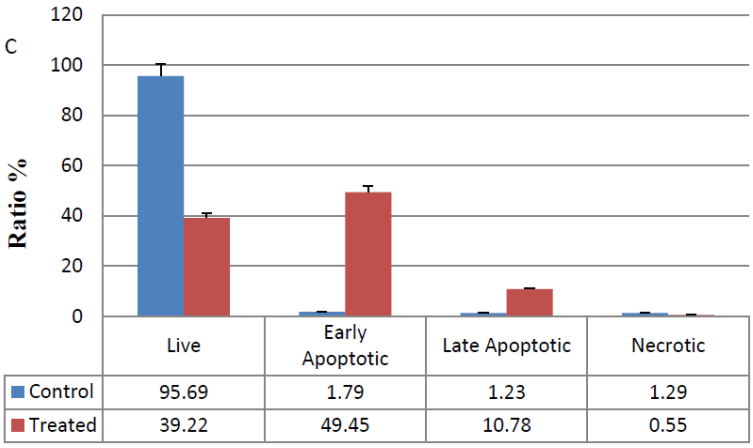
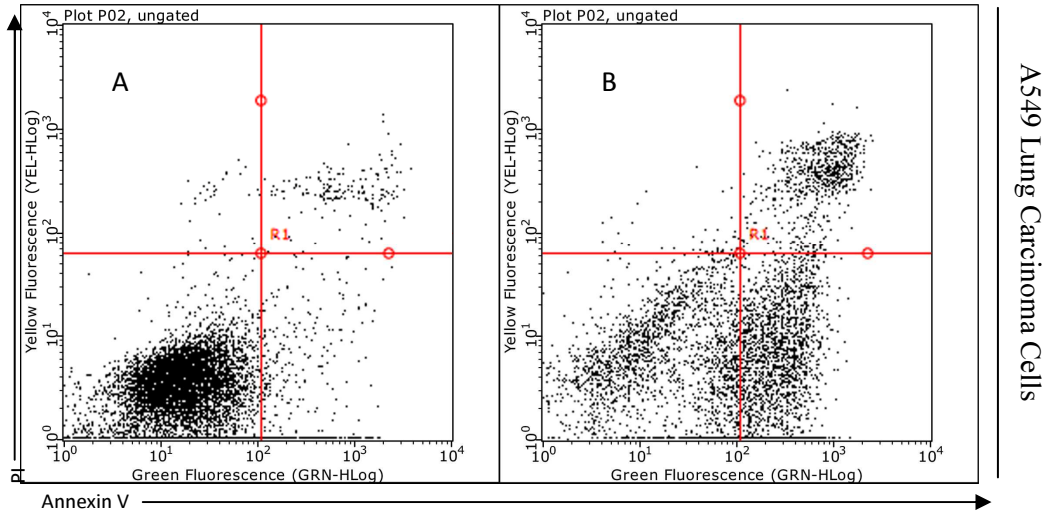


Fig. 2: BC-treated B16F10 Melanoma cells and their respective controls were stained by Annexin-V–FITC and Propidium Iodide (PI) and analyzed by flow cytometry. (A) Represents the B16F10 melanoma cells before BC treatment and acts as a control. (B) Represents B16F10 melanoma cells after BC treatment. (C) Bar chart comparing the ratio % between untreated and BC-treated B16F10 melanoma cells



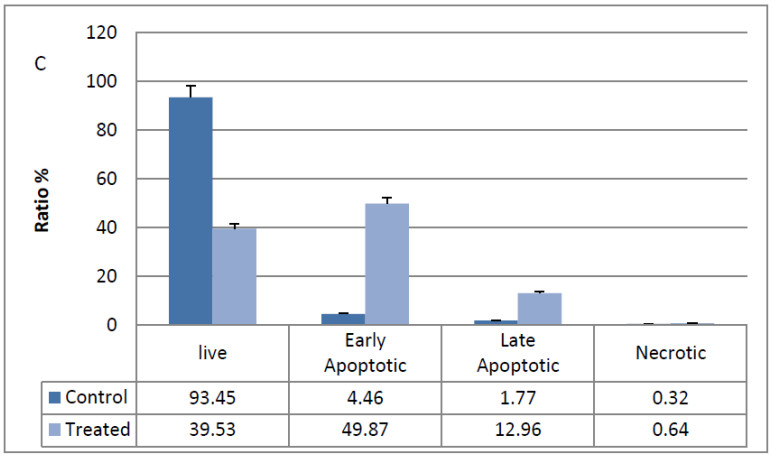


Fig. 3: BC-treated A549 human lung carcinoma cells and their respective controls were stained by annexin-V–FITC and Propidium Iodide (PI) and analyzed by flow cytometry. (A) Represents the A549 lung carcinoma cells before BC treatment and acts as a control. (B) Represents A549 lung carcinoma cells after BC treatment. (C) Bar chart comparing the ratio % between the ntreated and BC-treated A549 lung carcinoma cells.

In Vivo Results

Immune Response after BC Treatment

Neter *et al*. (1956) reported a passive hemagglutination method directed against polysaccharides found in a molecule and Boyden (1951) reported a passive hemagglutination directed against proteins found in a molecule. Using both Neter's and Boyden's method of passive hemagglutination, when compared to the normal serum control tube, BC- treated mice sera failed to cause agglutination of the sensitized erythrocytes in all the serially diluted tubes (1:10-1:20480) as it was indicated by the absence of diffused film at the bottom of the tubes.

Discussion

Prudden's systematic analysis of Bovine Cartilage's anti-inflammatory activity paved the way to discover different mechanisms by which BC acts on tumor cells. In our pervious study we investigated some of its mechanisms of action against cancer cells (Tanelian *et al*., 2016). The aim of this study was to evaluate the *in vitro* anti-tumor activity of BC, determine its mode of action on tumor cells and assess its immunogenicity.

Previously, we showed that BC has direct anti-tumor activity against mouse B16F10 melanoma cells (Tanelian *et al*., 2016). To further underscore this direct anti-tumor effect, three different human tumor cells MDA-MB-231, A549 and PC3 cells were incubated separately with increasing concentrations of BC for 48 h. In all three human tumor cells, significant dose-dependent decrease in their viability was noted, reaching a total eradication of tumor cells at 5000 μg mL^{-1} of BC. These results were consistent with our pervious outcome and with Durie *et al*. (1985) who, using the same BC concentrations demonstrated the anti-proliferative activity of BC against several human tumor cell lines.

In an attempt to identify the mode by which BC exerts its direct anti-tumor effect on tumor cells, two different tumor cells, the mouse B16F10 melanoma cells and the human A549 lung carcinoma cells were exposed to BC treatment for 48 h. The flow cytometry results indicated that both BC-treated tumor cell types underwent apoptosis. The obtained results imply that the anti-tumor activity of BC is not restricted to a certain cell type and is the same across different species. In a similar study Prudden (1964) concluded that the effect of repair stimulating factor found in cartilage is not species specific.

Finally, in our previous study (Tanelian *et al*., 2016), we showed that, as Prudden stated, BC has an important role as a biological modifier. However, several questions were raised whether or not BC provokes immune response against itself. For this purpose, passive hemagglutination was performed and negative results were obtained. Our results are also confirmed by (Langer and Gross, 1974) where they classify cartilage as an "immunoprivileged" tissue (Langer *et al*., 1976).

Conclusion

The direct anti-tumor activity of BC on tumor cells seems to be through inducing apoptosis and this mechanism of action is the same across different species and cell types. Moreover, it appears that BC does not provoke the host immune response against itself.

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Authors' Contributions

Arax Tanelian: Contributed to the conception and design of this study, acquisition of laboratory data, data analysis and/or interpretation, drafting and /or critical revision of the manuscript and approved this final version.

Alexander M. Abdelnoor: Contributed to the conception and design of this study, drafting and/or critical revision of the manuscript and approved this final version.

Conflict of Interest

The authors declare that they have no conflict of interests.

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