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Title: Anticancer properties of chitosan on human melanoma are cell line dependent

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Keywords: Chitosan; melanoma; apoptosis

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Abstract: Purpose: Chitosan, a natural macromolecule, is widely used in medical and pharmaceutical fields because of its distinctive properties such as bactericide, fungicide and above all its antitumor effects. Although its antitumor activity against different types of cancer had been previously described, its mechanism of action was not fully understood. Materials and Methods: Coating of chitosan has been used in cell cultures with A375, SKMEL28 and RPMI7951 cell lines. Adherence, proliferation and apoptosis were investigated. Results: Our results revealed that whereas chitosan decreased adhesion of primary melanoma A375 cell line and decreased proliferation of primary melanoma SKMEL28 cell line, it had potent pro-apoptotic effects against RPMI7951, a metastatic melanoma cell line. In these latter cells, inhibition of specific caspases confirmed that apoptosis was effected through the mitochondrial pathway and Western blot analyses showed that chitosan induced an up regulation of pro-apoptotic molecules such as Bax and a down regulation of anti-apoptotic proteins like Bcl-2 and Bcl-XL. More interestingly, chitosan exposure induced an exposition of a greater number of CD95 receptor at RPMI7951 surface, making them more susceptible to FasL-induced apoptosis. Conclusion: Our results indicate that chitosan could be a promising agent for further evaluations in antitumor treatments targeting melanoma.

***Reviewer Suggestions**

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The authors want to thank Dr Kennedy and the reviewer for their time and attention.

Reviewer #1: In this manuscript, the authors cultured three human melanoma cell lines onto chitosan coated surface and studied the cell adhesion, proliferation and apoptosis.

Major questions and comments

1. Does such anticancer effect of chitosan depend on the concentration?
2. Chitosan does not contain any cell adhesion motif and thus cannot well support cell attachment and proliferation. Do the authors use any control (non cancer cells, like fibroblasts) to test the cell response and behavior? This is very important. If the coated chitosan also reduced the normal cell attachment and proliferation, the anticancer effect is actually quite questionable and the whole mechanism is not convincing at all.
3. All the tests were conducted within 48 h. How about long term effects?

Point 1: This point is very interesting, notably to verify if lower doses could also affect melanoma cells and especially the RPMI7951 metastatic cancer cells. It could also be pertinent to minimize adverse effect on healthy cells. We have evaluated the effect of chitosan concentration on RPMI7951 cell line and normal primary human dermal fibroblasts and it is reported on Fig.5C (for caspase-3 activity) and D (for cell count using WST-1 test).

Point 2: Previous the first submission, we had done tests to verify whether apoptosis induction was detectable on fibroblasts but not extensively. Thus, the remark of the reviewer was a chance to improve this paper. Chitosan effect was assessed on adhesion, proliferation and apoptosis of normal primary human dermal fibroblasts. As depicted in fig.5A, apoptotic morphology was not seen when fibroblasts were cultured on chitosan coating as well as no/low caspase activation (Fig.5C, even at high doses of Chitosan, and Fig.5F, even when cultures were continued for 6 days). However, it could be noted that the cell number was reduced (Fig.5D and E), probably as a result of a reduced proliferation. In vivo, because fibroblasts, unlike cancer cells, have a very slow proliferation rate, chitosan should have a low action on these cells. Cell attachment was tested (Fig.5B) and, even if a delay could be observed in the presence of chitosan after 6 hours, virtually all fibroblasts had adhered on the plate.

Point 3: Long term cultures (here 6 days) have been tested. Caspase activation increased substantially when RPMI7951 were seeded onto chitosan coated plates but such a phenomenon could not be observed when fibroblasts were used (Fig5.F). Chitosan also induced caspase-3 activation into A375 cells but only at day 6 and the effect remains unclear because if cell count decreased it was in the same way as in controls (Did the cells become too numerous in the plate and did exhaust the culture medium ?).

To respond to the comments, a new figure was added (now Figure 5) and text was modified to take account of the reviewer questions.

Abstract

Purpose: Chitosan, a natural macromolecule, is widely used in medical and pharmaceutical fields because of its distinctive properties such as bactericide, fungicide and above all its antitumor effects. Although its antitumor activity against different types of cancer had been previously described, its mechanism of action was not fully understood. Materials and Methods: Coating of chitosan has been used in cell cultures with A375, SKMEL28, and RPMI7951 cell lines. Adherence, proliferation and apoptosis were investigated. Results: Our results revealed that whereas chitosan decreased adhesion of primary melanoma A375 cell line and decreased proliferation of primary melanoma SKMEL28 cell line, it had potent pro-apoptotic effects against RPMI7951, a metastatic melanoma cell line. In these latter cells, inhibition of specific caspases confirmed that apoptosis was effected through the mitochondrial pathway and Western blot analyses showed that chitosan induced an up regulation of pro-apoptotic molecules such as Bax and a down regulation of anti-apoptotic proteins like Bcl-2 and Bcl-XL. More interestingly, chitosan exposure induced an exposition of a greater number of CD95 receptor at RPMI7951 surface, making them more susceptible to FasL-induced apoptosis. Conclusion: Our results indicate that chitosan could be a promising agent for further evaluations in antitumor treatments targeting melanoma.

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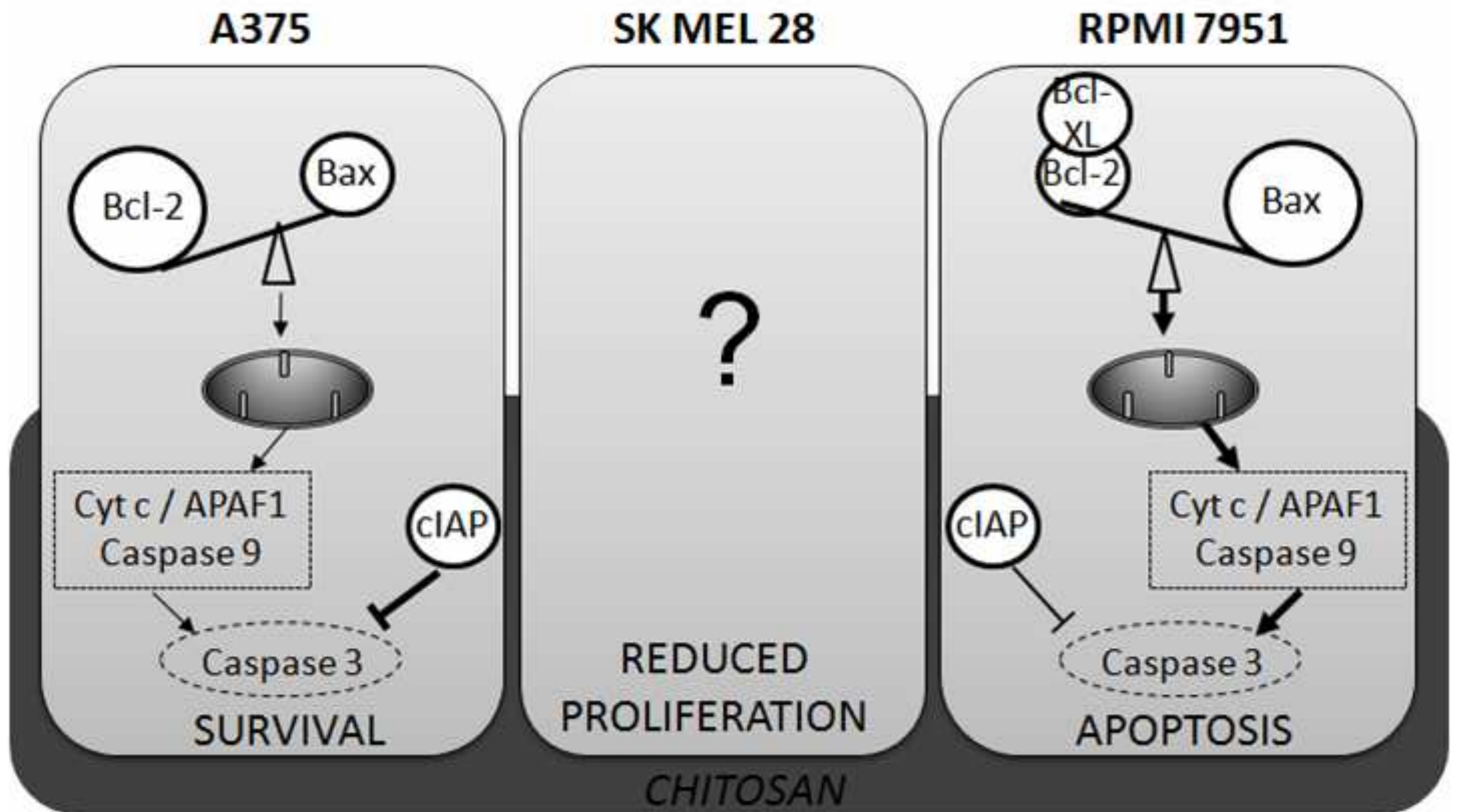

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Manuscript Title: Anticancer properties of chitosan on human melanoma are cell line dependent

Original article

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Running title: Anticancer effects of chitosan on melanoma depend on cell lines

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Conflict of interest: The authors declare that they have no conflict of interest.

Abstract

Purpose: Chitosan, a natural macromolecule, is widely used in medical and pharmaceutical fields because of its distinctive properties such as bactericide, fungicide and above all its antitumor effects. Although its antitumor activity against different types of cancer had been previously described, its mechanism of action was not fully understood. Materials and Methods: Coating of chitosan has been used in cell cultures with A375, SKMEL28, and RPMI7951 cell lines. Adherence, proliferation and apoptosis were investigated. Results: Our results revealed that whereas chitosan decreased adhesion of primary melanoma A375 cell line and decreased proliferation of primary melanoma SKMEL28 cell line, it had potent pro-apoptotic effects against RPMI7951, a metastatic melanoma cell line. In these latter cells, inhibition of specific caspases confirmed that apoptosis was effected through the mitochondrial pathway and Western blot analyses showed that chitosan induced an up regulation of pro-apoptotic molecules such as Bax and a down regulation of anti-apoptotic proteins like Bcl-2 and Bcl-XL. More interestingly, chitosan exposure induced an exposition of a greater number of CD95 receptor at RPMI7951 surface, making them more susceptible to FasL-induced apoptosis. Conclusion: Our results indicate that chitosan could be a promising agent for further evaluations in antitumor treatments targeting melanoma.

Key words: Chitosan, melanoma, apoptosis

Introduction

Chitin, the second most abundant natural polymer on earth after cellulose, is present in fungal cell walls and in the exoskeleton of arthropods like crustaceans [1-2]. Chitosan, an N-deacetylated derivative of chitin, is one of the most abundant, renewable, non-toxic, non-immunogenic, biodegradable and biocompatible carbohydrate polymers [3-4]. Chitosan is also known to have, per se, several biological activities [5-10]. Due to its properties described above, chitosan has been widely studied and developed for a variety of biomedical applications including wound dressings [11-12], hemostatic dressings [13-14] and antitumor drug delivery systems [15-18].

Besides this, chitosan also seems to have anticancer activities *per se* [17]. Although these well known properties have been frequently described, relatively few experiments seem to have been conducted to analyze and understand the chitosan signal-transducing mechanism involved in its anticancer activity. The action of chitosan as an anti-proliferative agent has been previously reported, even if the underlying molecular mechanism has yet not been fully investigated. Briefly, the anticancer properties of chitosan have been explained by an antiproliferative action in human gastric carcinoma cell line MGC803 cell [19], as well as in some human breast cancer cell lines [20] or in a human monocytic leukemia cell line, THP-1 [21]; by a necrotic action in various tumor cell lines [19, 22-23]; or by an apoptotic effect in several other cancer cell lines [24-25]. More specifically, Takimoto *et al.* showed that chitosan induced apoptosis by modulating death receptor expression and activating caspase-8 on human bladder tumor cells [25] while Hasegawa *et al.* demonstrated that chitosan induced apoptotic death of bladder tumor cells *via* caspase-3 activation [24]. On the other hand, Qi *et al.* demonstrated that an antitumor mechanism induced by chitosan in human hepatocellular carcinoma was mediated by the neutralization of cell surface charge, decrease of mitochondrial membrane potential and induction of membrane lipid peroxidation [26]. The pro-apoptotic mechanism of chitosan, which should be important for clinical applications, remains poorly understood even though *in vitro* and *in vivo* cytotoxic effects against various tumor cell lines were verified.

Melanoma is a devastating skin cancer. Worldwide, it affects 160,000 people a year and kills 48,000 of them. A panel of treatments ranges from surgeries to immunotherapies but without efficient results on metastases appearing in latter stages of the most aggressive cancers. Only a few studies were

conducted to investigate chitosan effects on melanoma cells and none delved into its potential anticancer activity. Chitosan and its derivatives were demonstrated to be able to decrease the invasive activity of murine [27] and human [28] melanoma cells, and to inhibit tumor angiogenesis induced by murine melanoma cells [29]. The aim of the present study was to examine chitosan antitumor effects on three distinct human melanoma cell lines: two from a primary site and one from a metastatic site. Our results suggested that chitosan could be useful in the antitumor treatment of several primary or metastatic melanomas.

Materials and methods

Cell culture. Melanoma cell lines were purchased from A.T.C.C. The A375 (A.T.C.C. # CRL-1619) and the SKMEL28 (A.T.C.C. # HTB-72) are primary melanoma and were derived from skin, whereas RPMI7951 (A.T.C.C. # HTB-66) was derived from a metastatic lymph node. All cells were grown in Dulbecco-Vogt modification of Eagle's medium (DMEM) (Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal calf serum (FCS) (HyClone, Logan, UT, USA), 100 U/mL penicillin (Sigma-Aldrich, Oakville, ON, Canada), 25 μg/mL gentamicin (Schering, Pointe Claire, Canada) in 8% CO2 at 37°C. For some experiments, we used these three melanoma cell lines stably transduced with DsRed sequence as described in [30]. Fibroblasts (Fb) were isolated from a skin biopsy of a 25 year-old French-Canadian woman as described in [30]. All procedures involving patient biopsy were conducted according to the Helsinki Declaration and were approved by the local Research Ethical Committee. The informed consent of donor was obtained.

Chitosan treatment. A highly viscous chitosan, purchased from Fluka Biochemica (Sigma), was dissolved in 0.1% acetic acid (AcOH) (EMD Biosciences, Gibbstown, NJ, USA). Culture wells were coated with 2 mg/ml chitosan in 0.1% acetic acid (AcOH) or with 0.1% AcOH alone. Briefly, solutions were distributed in an excess volume into each well to ensure the entire surface area was covered. Plates were placed at 4°C overnight. The next day, prior to plating melanoma cells, remaining chitosan or acetic acid solution was aspirated.

Cell viability, attachment kinetic and growth rate determination. A colorimetric MTT assay was adapted from Mosmann [31] to quantify cell viability after 2 days of culture onto acetic acid or

chitosan in 96-wells plates. Attachment kinetic and growth rate were determined using DsRed melanoma cells plated onto AcOH or chitosan-coated plates. For attachment kinetics, supernatants were collected 2h, 4h, 6h and 8h after seeding to detect non-adhered cell fluorescence. For growth rate curve, adherent cells were washed with PBS 24h, 48h and 72h after seeding. Briefly, supernatant or plates were flash-frozen at -80°C, thawed and distributed in black 96-well microplates. Fluorescence was read with a Varioskan fluorometer (Thermo Scientific, Milford, MA, USA). Proportional relation between fluorescence and number of cells was verified using serial-dilutions of a known cell number. Doubling time was determined by the classical formula, D=Ln(2)/g were g is the exponential coefficient of the slope. In experiments involving Fb (non fluorescent cell population) alternative techniques were used. For adhesion, RPMI7951 and Fb cells were plated in a 12-well plate (coated with 0.1 % AcOH or 2mg/ml chitosan in 0.1% AcOH) at 70% of confluency. Cells were harvested at the indicated time. After a ten fold dilution in an isotonic solution (Isoton), they were counted in a Z1 cell counter (Coulter Beckmann). For cell viability measurement, WST-1 (Clontech) was used following the manufacturer instructions. Cells were plated at 70 % confluency plated onto AcOH or chitosan-coated plates. In dose-response experiments, a range of 0.5 to 4 mg/ml chitosan was used and WST-1 added after 48 hours. In long-term experiments, WST-1 was added at the indicated time.

Proliferation rate. Proliferation rate was examined with 5-bromodeoxyuridine (BrdU)-pulsed cultures as previously described [32]. Briefly, melanoma cells were plated onto AcOH or chitosan in culture medium containing 10 µM BrdU for 24h. Cultures were then washed two times with fresh medium and incubated at 37°C for an additional 24h. Incorporated BrdU was detected by immunofluorescence with a primary mouse monoclonal antibody anti-BrdU (1/5, BD Pharmingen, Mississauga, ON, Canada), detected by an alexa 594-conjugated sheep anti-mouse (Molecular Probes, Eugene, OR, USA) mixed with Hoechst 33258 (Sigma). The number of BrdU (mitosis marker) positive nuclei was counted in 4 independent fields per glass slide.

Apoptosis induction. In order to activate the death receptor pathway, melanoma cells were incubated for 4.5 hours with DMEM 0.5% FCS + 20 ng/ml of Fc:FasL_3 (kind gift from Dr Pascal Schneider, Lausanne University, Switzerland [33]). Apoptosis *via* the mitochondrial pathway was induced with 100 ng/ml staurosporine (STS, Sigma) for 5.5 hours incubation. Chitosan was used at 2 mg/ml as

described above. Appropriate controls were simultaneously performed for each treatment.

Caspases activity measurement. Caspases-3, -8 and -9 distinct activities were measured in cell lysates using three distinct ApoAlert Caspase assay kits (Clontech, Palo Alto, CA, USA), according to the manufacturer's instructions. Total protein concentration in each cell lysates was determined using microBCA protein assay reagent kit from Pierce (Rockford, IL, USA). Plates were read on a Varioskan fluorometer (Thermo Scientific). Caspase activity, correlated with the apoptotic rate, was reported as fluorescence unit (Fu)/min/mg protein [34].

Specific inhibition of apoptosis. DsRed melanoma cells RPMI7951 were plated onto acetic acid or chitosan-coated plates at a density of 50.000 cells/cm^2 in DMEM + 0.5% FCS with or without caspase-8 inhibitor (Ac-IETD-CHO, 100 µM) and caspase-9 inhibitor (Ac-LEHD-CHO, 100 µM) (Enzo Lifesciences, Brockville, ON, Canada). Then, cells were cultivated for 16h For positive controls, RPMI7951 cells were cultivated for 16h on uncoated wells and apoptosis was induced by addition of 20 ng/ml of Fc:$FasL_3$ or 100 ng/ml of STS. After 24h, supernatants and attached cells were harvested separately and flash-frozen at -80°C. Fluorescence was evaluated using Varioskan fluorometer and the percentage of apoptosis was determined by dividing DsRed fluorescence in supernatants by the total fluorescence (supernatant + attached cells).

Protein extraction and Western blot analysis. Cells cultured for 48h onto chitosan-coating were harvested, washed, lysed, sonicated and proteins concentration was determined. Proteins (10 µg) were boiled in β-mercaptoethanol for 5 min, separated by SDS/PAGE (12% gels) and transferred onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). Non-specific binding sites were blocked for 1h at room temperature with 5% (w/v) fat-free skimmed milk powder before an overnight incubation at 4°C with specific antibodies: mouse monoclonal anti-Poly(ADP-Ribose) Polymerase (PARP) (Calbiochem; Ab-2 C-2-10), rabbit anti-p-Akt 1/2/3 (Santa Cruz biotechnology, Santa Cruz, CA, USA; sc-7985-R), rabbit anti-Akt 1/2/3 (Santa Cruz biotechnology; sc-8312), mouse anti-Bcl-2 oncoprotein (Boehringer Mannheim, Laval, QC, Canada; clone 124), mouse anti-Bad (Transduction laboratories, Mississauga, ON, Canada; B31420), mouse anti-Bcl-x_L (Santa Cruz biotechnology; sc-8392), rabbit anti-Bax (Cedarlane, Hornby, ON, Canada; AAS-040), goat anti-c-IAP 1/2 (Santa Cruz biotechnology; sc-12410) or with a mouse monoclonal anti-actin (Cedarlane, clone C4) antibody as a

loading control. Primary antibodies were detected with appropriate HRP (horseradish peroxidase)-conjugated anti-mouse, anti-rabbit antibodies (Pierce) or anti-goat antibody (Santa Cruz biotechnology). Blots were revealed using SuperSignal West Dura Extended Duration Substrate (Pierce) by autoradiography. After film scanning, Image J software (NIH, Bethesda, MD, USA) was used to estimate the relative amount of each protein.

Extracellular Fas receptor (CD95) detection. Freshly trypsinized melanoma cells were harvested in PBS containing 20 mM EDTA, incubated with an anti-CD95 monoclonal mouse antibody (R&D Systems, ON, Canada) or with isotypic IgG2a monoclonal mouse antibody (DAKO, ON, Canada), then washed and incubated with secondary anti-mouse phycoerytrin-conjugated antibody (Jackson Laboratories, PA, USA). Cells were washed before analysis by flow cytometry using FACScalibur (BD Biosciences).

Fas-specific apoptosis evaluation on coated surface. RPMI7951-DsRed cells were seeded onto AcOH or chitosan-coated plate. After 24h, apoptosis was induced with a low dose of Fc:FasL_3 (1 ng/ml). Apoptosis was evaluated as previously described. To determine the specific part of Fc:FasL_3 in apoptosis when cells were cultivated onto chitosan coating, the formula from Baumler e*t al* was applied [35]: % Fc:FasL_3-specific apoptosis in chitosan-coated conditions = [(%Fc:FasL_3+chitosan apoptosis - % chitosan apoptosis)/(100 - % chitosan apoptosis)]x100.

Statistical analysis. Differences between values were assessed by bilateral Student's t-test. All data were expressed as mean ± standard deviation (SD), and overall statistical significance was set at $p < 0.05$.

Results and Discussion

Chitosan reduced viable human melanoma cell number. Microscopic observations of cultures on chitosan-coated surface of primary melanoma cell lines A375 and SKMEL28, and metastatic melanoma cell line RPMI7951 showed a reduction of viable cell number compared to control, i.e. acetic acid-coated surface (Fig.1). Indeed, the upper panel of Fig.1A showed a typical result obtained when melanoma cell lines were cultivated for 48h on a surface coated with acetic acid (AcOH, control) or chitosan (Chitosan) and then treated with MTT. Mitochondrial enzymatic activity was

reduced by 28±2% for A375, 42±1.5% for SKMEL28 and 58±1.5% for RPMI7951 when compared with control (Fig.1A lower panel). Data obtained by the MTT test can result from variations in cell attachment properties, in cell proliferation, in cell death or in mitochondrial metabolism. Accordingly, further tests were then needed to account for these results.

Chitosan modified attachment properties of A375 melanoma cell line but not SKMEL28 and RPMI7951. Because chitosan did not present adhesion motifs for cell adhesion, we verified whether chitosan coating resulted in a modification of human melanoma cell lines ability to adhere/attach onto chitosan-coated surfaces. We took advantage of newly generated melanoma cell lines stably expressing DsRed to evaluate the number of living cells [30]. This labeling did not affect growth rate, proliferation or apoptosis sensitivity (data not shown). DsRed fluorescence correlated closely with cell number (Fig.1B, upper left panel). Attachment kinetics were monitored using the fluorescent properties of floating cells. As demonstrated in Fig.1B upper right panel, adhesion of A375 was significantly reduced from 90% to 73% whereas adhesion of SKMEL28 (Fig.1B, lower left panel) and RPMI7951 (Fig.1B, lower right panel) were not significantly different in the presence or absence of chitosan coating.

Chitosan reduced growth rate of SKMEL28 and RPMI7951 but not A375. After verifying the attachment properties, we investigated the growth rate. The doubling time was calculated from growth curves. As depicted in Fig.2A, chitosan significantly increased the doubling time for SKMEL28 (29.0h *vs* 30.5) and RPMI7951 (30.8h *vs* 34.4), whereas no significant change was noticed for A375 cell line (22.2h *vs* 22.1). Such an increase of doubling time, particularly in metastatic RPMI7951 cell line (+11.7%) could have a huge impact in limiting cancer progression. As growth rate could result from proliferation or cell death, other tests were thus required to investigate the mechanisms underlying this action of chitosan.

Chitosan reduced SKMEL28 proliferation rate but not RPMI7951. One hypothesis to explain the increased doubling time observed previously for two cell lines could be a reduction in the proliferation rate in the presence of chitosan. After a 24h pulse of BrdU, cells were incubated for another 24h and BrdU incorporation was analyzed in order to determine the proliferation rate of the both melanoma cell cultures which presented a difference in doubling time (Fig.2B). SKMEL28 cells

showed a significant reduction of their proliferation rate when cultivated on a chitosan coating, whereas RPMI7951 maintained their proliferation rate regardless of the coating. Thus, the increased doubling time induced by chitosan could be easily explained by a lower proliferation rate for SKMEL28 but not for RPMI7951. For this latter cell line, a decrease in viable cell number could be an explanation for the increased doubling time in the presence of chitosan.

Chitosan induced apoptosis in SK MEL 28 and RPMI7951 but not in A375 cell lines. Whereas variations in adhesion and proliferation properties appeared to be reasonable explanations for the reduction in cell number for A375 and SKMEL28, respectively, no such mechanistic answer was found for RPMI7951 when cultured on chitosan. Melanoma cells death thus remained a possibility. Effector caspase-3 specific activity, measured in cell lysates from melanoma cells cultured on a chitosan coating, largely increased over time for RPMI7951, slightly for SKMEL28 but was not detectable in A375 cultures (Fig.3A). PARP is a natural substrate of activated caspase-3. So, as expected, after cells were seeded onto chitosan coating, cleavage of PARP was found to be increased in SKMEL28 and RPMI7951 cell lines (data not shown). Caspase-3 activation can be set in motion through two main pathways: extrinsic (death receptor) pathway [36] or intrinsic (mitochondrial) pathway [37]. In order to design an anticancer drug which could potentiate the effect of chitosan on melanoma cells, it would be important to know which pathway is involved in chitosan induced apoptosis.

RPMI7951 were type I cells for Fas-induced apoptosis. In order to further elucidate the mechanism through which chitosan induced apoptosis, we verified which type of Fas-induced apoptosis was effected in the RPMI7951 cell line. There are two distinct modes of death receptor apoptosis regulation. Type I cells die by the canonical way (*i.e* DISC formation and caspase-8 activation) whereas type II cells die through activation of the mitochondria process *via* Bid truncation and subsequent caspase-9 activation [38]. Thus, after Fas activation, type I cells are characterized by sequential activation of caspase-8, -3 and -9 (and possibly back activation of caspase-8 by caspase-3) whereas in type II cells, caspase-9 is activated first, followed by caspase-3 and -8. Fig.3B shows that Fas activation by the Fc:$FasL_3$ extrinsic inducer of apoptosis led to activation of procaspase-8 (2.2h with a second activation at 5.5h), procaspase-3 (3.5h) and then procaspase-9 (4.5h). Thus, we

concluded that RPMI7951 were type I cells and had a functional death receptor pathway going through caspase-8 activation.

Apoptotic profile of human melanoma cell lines. Fc:FasL_3 is a powerful recombinant protein mimicking the effect of FasL trimers which induce apoptosis mediated by death receptor [33]. Staurosporine (STS) induces mitochondrial apoptosis by blocking phosphorylation of Bad by Akt and its subsequent sequestration and inactivation by 14-3-3 [39]. These two apoptotic inductors were used to define the response profile of melanoma cells to pro-apoptotic agents (Fig.3C). When treated with Fc:FasL_3, caspase-3 was activated A375 and RPMI7951 cell lines but not in SKMEL28. When treated with STS, caspase-3 was weakly activated in A375 and SKMEL28 cell line but strongly activated in RPMI7951. Interestingly, chitosan coating induced apoptosis in a similar profile as STS: no activation of caspase-3 in A375, weak activation in SK MEL28 and a strong activation in RPMI7951. Indeed, the SKMEL28 cell line was unable to respond to Fas activation but was sensitive to the induction of apoptosis through STS as well as to chitosan-induced apoptosis. The A375 cell line thus appears to be insensitive to chitosan-induced apoptosis regardless of its sensitivity to Fas. Finally, all three tested molecules induced apoptosis in the RPMI7951 cell line. Thus, both apoptosis pathways were functional in this cell line.

Chitosan induced apoptosis through pro-caspase-9 activation in RPMI7951 cells. The results presented above strongly suggested that chitosan-induced apoptosis in RPMI7951 went through the mitochondrial pathway. In order to further investigate this point, inhibition of apical caspases, namely caspase-8 for the death receptors pathway and caspase-9 for the mitochondrial pathway, was done during chitosan-induced apoptosis. To avoid potential side effects of caspase inhibitors on caspase-3 activity, we took advantage of the fluorescence of RPMI7951-DsRed cells to monitor apoptosis. Fc:FasL_3-induced apoptosis, as a positive control for death receptors pathway, and staurosporine-induced apoptosis, as a positive control for mitochondrial apoptosis, were tested in parallel with the pro-apoptotic effect of chitosan coating on RPMI7951 cells. Whereas Ac-LEHD-CHO, a caspase-9 inhibitor, reduced apoptosis in chitosan-induced apoptosis as it did with STS-induced apoptosis, Ac-IETD-CHO, a caspase-8 inhibitor, failed to cause the same effect, confirming chitosan activates pro-caspase-9 *via* the mitochondrial pathway but not pro-caspase-8 via the death receptors pathways (Fig.

4). Thus, we confirmed that apoptosis induced by chitosan in RPMI7951 metastatic cell line went through the mitochondrial pathway.

Normal human cells adhere onto a chitosan coating and did not undergo apoptosis. In order to investigate if chitosan could have anti-cancer cell-specific properties, human primary dermal fibroblasts were used. As evidenced in Fig.5A by photographs, fibroblasts were not morphologically affected by the chitosan coating. Adhesion of fibroblasts was delayed but not impaired (Fig.5B). Chitosan did not activate caspase, or few, in fibroblasts until exposed to a concentration of 4 mg/ml, whereas caspase-3 activity was obviously increased in RPMI7951 melanoma cell line (Fig.5C). Nevertheless, when the highest tested concentration of chitosan was used to coat cell culture surfaces, a reduced number of viable fibroblasts was observed compared to lower concentrations (Fig.5D). The same phenomenon was observed when culture was maintained until day 6. Whereas RPMI7951 cell count was notably decreased in chitosan-coated plates compared to control viable fibroblasts populations decreased in a similar manner (Fig.5E) but without activation of caspases-3 contrarily to RPMI7951 (Fig.5F). Such a slowdown in fibroblasts growth could be attributable to a decrease in proliferation rate because although they could adhere on chitosan-coated plate, no cell was seen to adopt an apoptotic morphology or caspase activation can be observed. Unlike cancer cells, in vivo fibroblasts embedded in extracellular matrix normally have little proliferative capacity, except in the case of a wound, and then should remain unaffected by a potent anti-proliferative action of chitosan. Interestingly, at day 6, A375 cell count decreased into chitosan-coated plates in a way similar to control whereas caspase activation was increased when chitosan coating was used.

Bax was up regulated while Bcl-2 and Bcl-XL were down regulated in chitosan-treated RPMI7951 cells. To pinpoint the potential mechanism through which chitosan induced apoptosis in the melanoma cell lines, several proteins involved in the mitochondrial pathway were evaluated by Western Blot by ImageJ software (Fig.6A). Histograms, resulting from analysis of Western blot films, revealed relative expression levels of protein expression from melanoma cells seeded on AcOH or chitosan-coated surface (normalized relatively to actin content or AKT for pAKT). In chitosan-exposed A375 cells Bax was strongly down regulated and c-IAP slightly upregulated, which was consistent with the absence of apoptosis in this melanoma cell line after seeding on chitosan-coated

surface (Fig.6B upper panel). The results for SKMEL28 cells were more nuanced since chitosan induced an upregulation of Bax but also of c-IAP, a strong inhibitor of caspases activation. We postulated that the effect of one compensated the effect of the other (Fig.6B mid panel), explaining the low induction of apoptosis in the presence of chitosan in that cell line. Anti-apoptotic proteins Bcl-2 and Bcl-XL were down regulated in chitosan-treated RPMI 7951 cells while pro-apoptotic protein Bax was up regulated (Fig.6B lower panel). The Bax/Bcl-2 expression level ratio increased 3 fold after RPMI7951 seeding on chitosan-coated surface and could explain this cell line's high sensitivity to the induction of apoptosis through the mitochondrial pathway (Fig.6C). By understanding the molecular response to chitosan in different cancer cell lines, improved antitumor treatments could be designed.

A375 and RPMI7951 cell lines displayed a high level of CD95/Fas receptor at their surface but SKMEL28 did no. Since some studies showed that death receptors were involved in chitosan-induced apoptosis [25], we wanted to establish the basal level of Fas receptor expressed on the cell surface of each cell line and whether it was modulated by chitosan exposure. Surface exposed Fas receptor was determined by flow cytometry without permeation of cells. As seen in Fig. 7A, A375 cells expressed the same amount of Fas receptors when cultivated onto vehicle or chitosan-coated surfaces. SKMEL28 cell line displayed a very low level of CD95 receptor. This observation was in accordance with its inability to respond to Fas activation (Fig.3C). RPMI7951 cells displayed more receptors than A375. Interestingly, chitosan induced an increase of Fas receptors expressed on the cell surface of RPMI7951 cells, the most sensitive to chitosan-induced apoptosis. This observation paved the way for further experiments to investigate if chitosan pre-treatment could enhance Fas therapy.

RPMI7951 cells seeded on chitosan-coated surface were more sensitive to Fas-induced apoptosis than controls. RPMI7951 cells sensitivity to Fas activation-induced apoptosis was tested in the presence or absence of chitosan coating (Fig.7B). To quantify apoptosis induced in cells cultured on different coatings, we evaluated the percentage of apoptosis specific to Fas with a modified formula from Baumler *et al* [35]. Whereas Fas-specific apoptosis from the control surface was 20.1±2.8%, it was 28.7±3.4% for the chitosan-coated surface group. Thus, chitosan increased RPMI7951 cell line

sensitivity to Fas-induced apoptosis by approximately 10%, which could have a great impact in the context of a repeated treatment.

In conclusion we have demonstrated that chitosan had distinct biological effects on three human melanoma cell lines (Fig.8). When grown on a chitosan coating, primary melanoma cells A375 display neither a proliferation decrease nor apoptosis induction, but their adhesion capacity was reduced. Nevertheless, the elucidation of the mechanisms that protect this cell line from apoptosis induction, i.e. decrease of Bax and increase of Bcl-2 and c-IAP, could open the way to adapted chemotherapeutic drugs design. In primary melanoma cells SKMEL28, apoptosis was induced at a low level by chitosan, probably due to the enhancement of c-IAP expression. More interestingly, for this cell line, chitosan reduced the proliferation rate (-15.1%) and consequently the growth rate of cancer cells, seriously impacting the long term growth of the tumor. It could be interesting, as a future goal, to investigate how to enhance mitochondrial apoptosis rate of these cells, since they did not express cell death receptors on their surface. Chitosan coating was able to induce *in vitro* a high level of apoptosis in metastatic melanoma cell line RPMI7951; This effect was concentration-dependent and increased with time. Caspase inhibitors experiments confirmed that chitosan induced apoptosis through the mitochondrial pathway in this cell line. Furthermore, chitosan induced an up regulation of mitochondrial pro-apoptotic protein Bax and a down regulation of anti-apoptotic proteins such as Bcl-2 and Bcl-XL. Interestingly, CD95/Fas receptor level was increasingly displayed on the RPMI7951 cell surface when these cells were exposed to chitosan. This increase of receptor number on the surface of the cells led to a higher sensitivity of RPMI7951 to Fas-mediated apoptosis. A negative side-effect on healthy cells could be excluded by results obtained by using fibroblasts where no capase activation could be observed. These promising results pave the way for the design of new efficient antitumor strategies against metastatic melanoma based on a non-toxic agent, namely chitosan, that could trigger both mitochondrial and death receptor apoptosis pathways. The use of chitosan, in combination or not with a death receptor activator, is easy to imagine due to its properties which render it an efficient and easy-to-handle drug carrier [40-41].

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Authors contributions Conception and design: S. Chabaud (all) and L. Gibot (all – Fig.4 and 5); Data Acquisition, analysis and interpretation: L. Gibot (all – Fig.4 and 5), S. Chabaud (all) and S. Bouhout (Fig.4); Manuscript writing: S. Chabaud and L. Gibot; Manuscript revision: V. Moulin, F.A. Auger and S. Bolduc. Study supervision: V. Moulin.

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Figure legends

Figure 1. Three human melanoma cell lines presented a reduced growth potential when cultivated onto chitosan not explainable by a reduced cell adhesion, except for A375 cell line. (A) Typical MTT viability test after 48h in presence (Chitosan) or absence (AcOH) of chitosan coating. Histograms showed the optical density, relative to control for each condition, which allow determining the number of viable cells. N=8. (B) Fluorescence measurement is a direct measure tightly correlated to the number of cells in culture. A375-DsRed, SKMEL28-DsRed and RPMI7951-DsRed melanoma cell lines were tested for their ability to adhere onto control (AcOH) or chitosan coating (Chitosan). Values were the mean of 3 experiments ± standard deviation. * = $p<0.05$

Figure 2. Chitosan reduced growth rate of SKMEL28 and RPMI7951 cells but only proliferation rate of SKMEL28 (A) Doubling times were calculated from growth curve for A375, SK MEL 28 and RPMI 7951 cells when cultivated onto control coating (AcOH, blank bars) or chitosan coating (Chitosan, black bars). N=3. (B) Incorporation of BrdU, a mitosis marker, when cells were cultivated onto control (blank bars) or chitosan (black bars) coating. N=3. * = $p<0.05$.

Figure 3. Chitosan induced apoptosis in RPMI7951, a cell line which possesses fully functional extrinsic and intrinsic apoptotic pathways. (A) Time course of caspase-3 activation after seeding melanoma cells onto a chitosan coating. N=3. * = $p<0.05$ for A375 *vs* SKMEL28, # = $p<0.05$ for SKMEL28 *vs* RPMI7951. (B) Determination of RPMI7951 cell type in regard to Fas-induced apoptosis. Kinetics of activation of apical initiator caspases, caspase-8 (C8, square) and -9 (C9, circles), and effector caspases, caspase-3 (C3, triangles) after Fas stimulation are used to determine the cell type. Due to different levels of enzymatic specific activity between caspases, activity is normalized with the highest value. N=3. Standard deviations were omitted to an easy reading of the figure but were negligible. (C) Caspase-3 specific activity profile for each melanoma cell line when induced with a Fas stimulation by Fc:FasL$_3$ (Fc:FasL$_3$), staurosporine (STS) or chitosan coating (Chitosan). N=3.

Figure 4. Chitosan-induced apoptosis in RPMI7951 was inhibited by Ac-LEHD-CHO like STS-induced apoptosis but not by Ac-IETD-CHO unlike Fas-induced apoptosis. RPMI7951 seeded in

uncoated wells were cultivated with or without caspase-8 inhibitor (Ac-IETD-CHO) and caspase-9 inhibitor (Ac-LEHD-CHO) before apoptosis induction with 20 ng/ml of Fc:FasL$_3$ (Fas) or 100 ng/ml Staurosporine (STS). RPMI7951 seeded onto chitosan coating were also cultivated with or without specific caspases inhibitors. Caspase-3 activation allowed quantifying induced apoptosis. N=8. * = $p<0.05$

Figure 5. Chitosan did not induce apoptosis in normal cells. Values are the mean of the indicated number of experiments ± standard deviation * = $p<0.05$

(A) Representative photographs of A375, RPMI7951 and dermal fibroblasts cultured for 48h onto AcOH (0 mg/ml) or Chitosan (2mg/ml) (B) RPMI7951 melanoma cell lines and dermal fibroblasts (FB) were tested for their ability to adhere onto control (AcOH) or chitosan coating (Chitosan). N=3. (C) Effect of the concentration of Chitosan on caspase-3 activation after seeding melanoma cells (RPMI) or fibroblasts (FB) onto a chitosan coating. N=3 (D) Effect of the concentration of Chitosan on viable cell number determined by WST-1 test after seeding melanoma cells (RPMI) or fibroblasts (FB) onto a chitosan coating. N=8 (E) Time course of caspase-3 activation after seeding melanoma cells (A375 or RPMI) or fibroblasts (FB) onto a chitosan coating. N=3 (F) Effect of a long term culture onto Chitosan coating on viable cell number determined by WST-1 test after seeding melanoma cells (A375 or RPMI) or fibroblasts (FB), N=12.

Figure 6. Mitochondrial apoptotic pathway proteins were modulated by chitosan. (A)After being seeded onto AcOH (-) or chitosan (+) coating, cells were harvested and resulting lysates were analyzed by Western blotting. (B) Histograms depicted protein expression level relative to actin content (or pAKT relative to AKT) for A372, SKMEL28 and RPMI7951 cells. (C) Bax to Bcl-2 ratio changes between AcOH and chitosan coated conditions for RPMI7951 cell line.

Figure 7. Chitosan sensitized RPMI7951 cells to Fas-induced apoptosis (A) Cell surface Fas receptor exposition was examined by flow cytometry technique. Geometric mean of labeling reflects the amount of receptors exposed to cell surface. N=3. (B) Fc:FasL$_3$ specific apoptosis when RPMI7951 cells where cultivated onto control or chitosan coating. N=6. * = $p<0.05$.

Figure 8. Recapitulating schema of chitosan coating observed biological effect on the three melanoma cell line. Bcl-2 family pro- and anti-apoptotic members play a key role in the control of

mitochondrial destabilization via multiple complexes in balance. Mitochondria releases several factors, then cytochrome C, APAF-1 and pro-caspase-9 associate and caspase-9 activation leads to caspase-3 effector activation and subsequent cell death (RPMI 7951). This latter activation could be inhibited by cIAP (A375). No molecular mechanism was elucidated for the reduction of proliferation of SK MEL 28.

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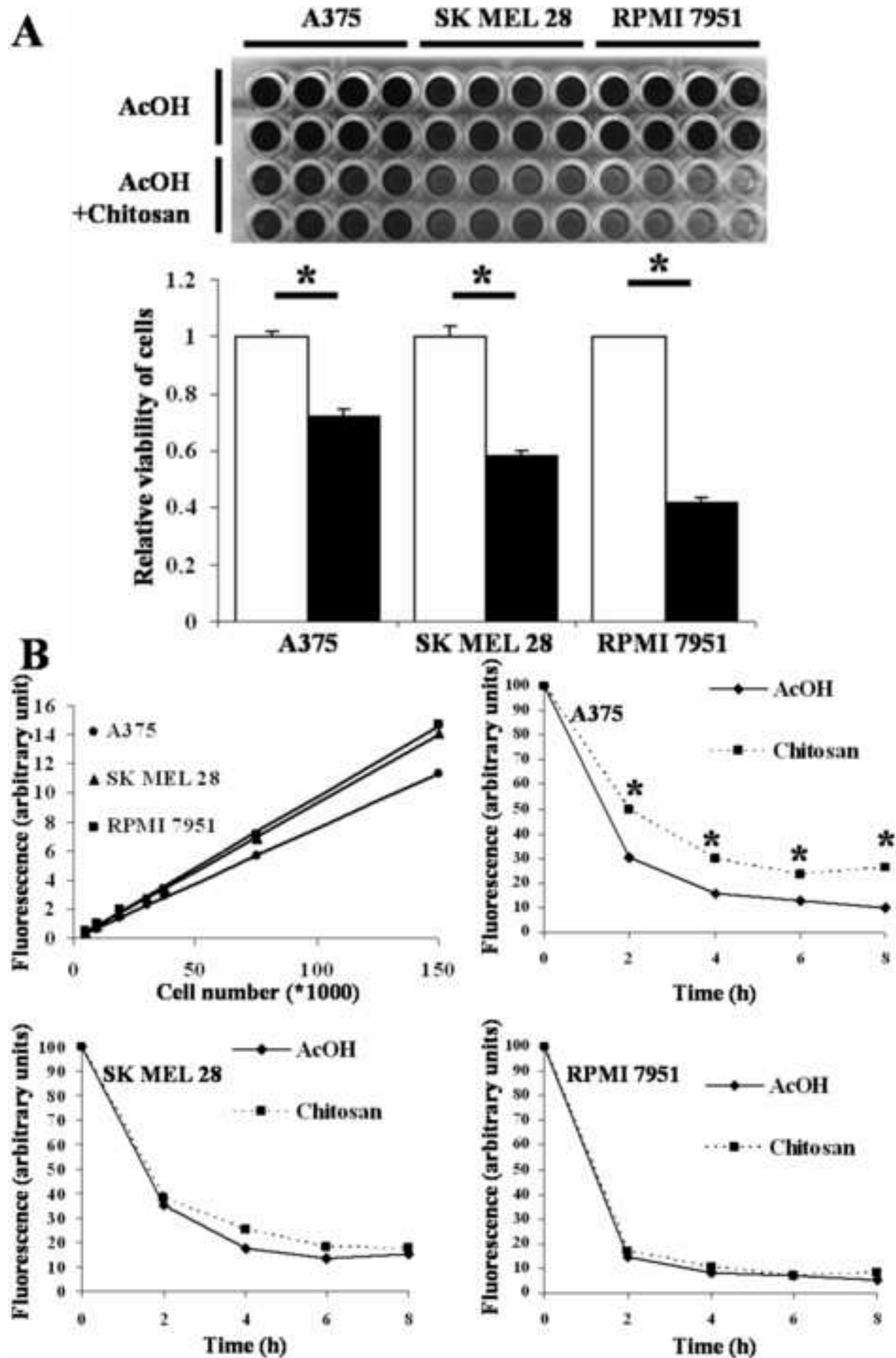

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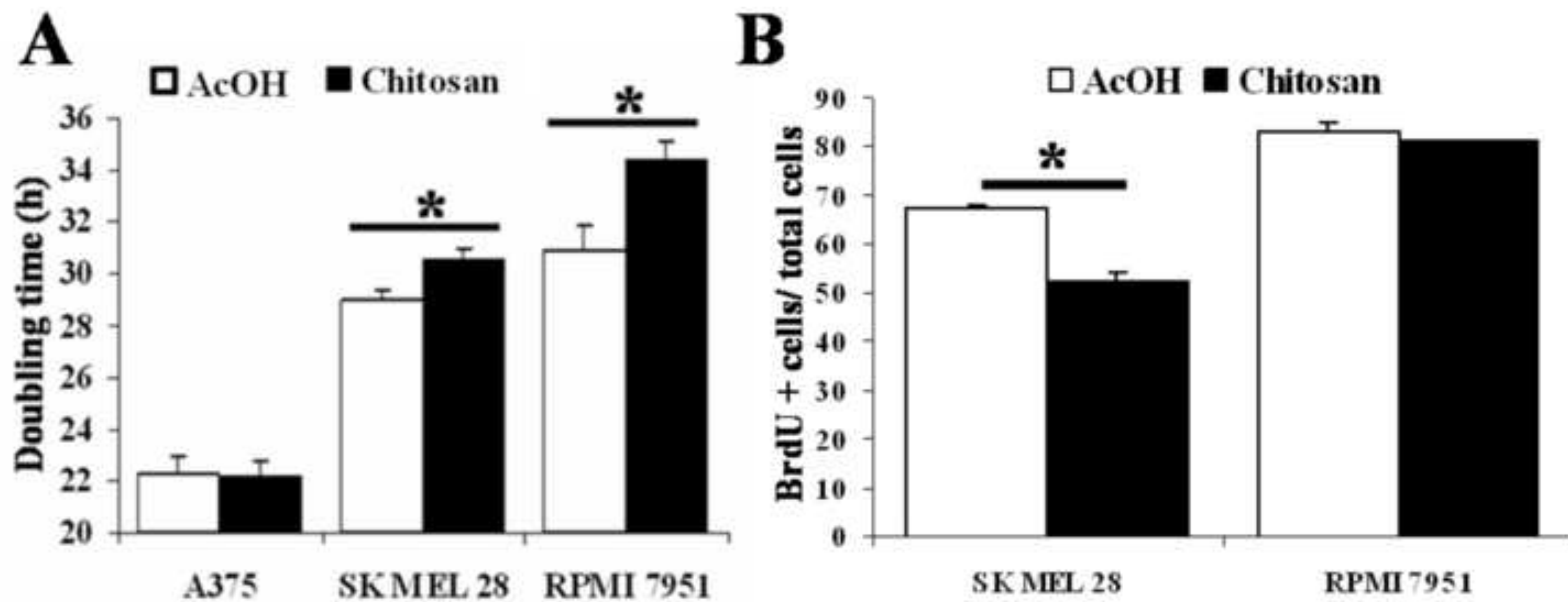

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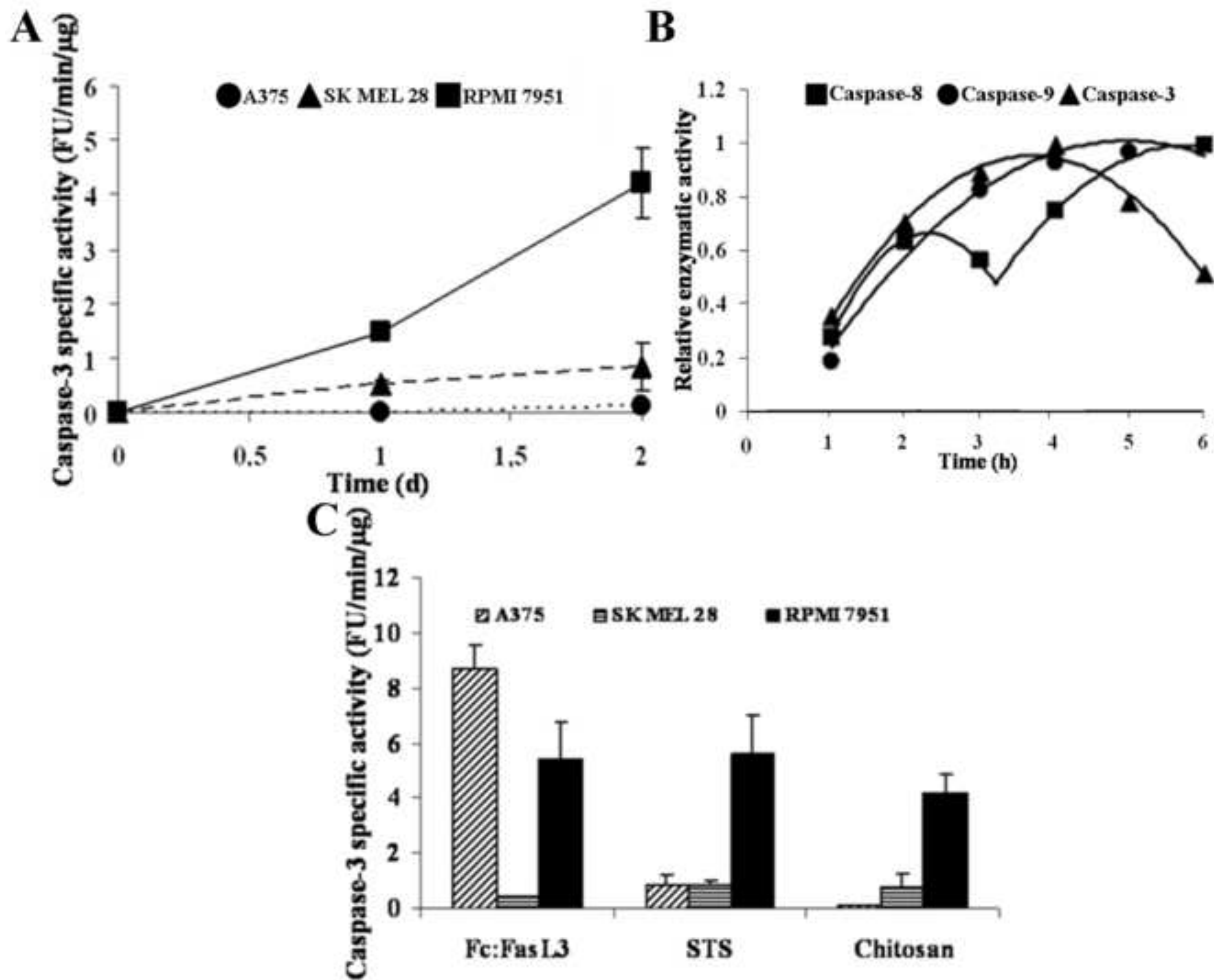

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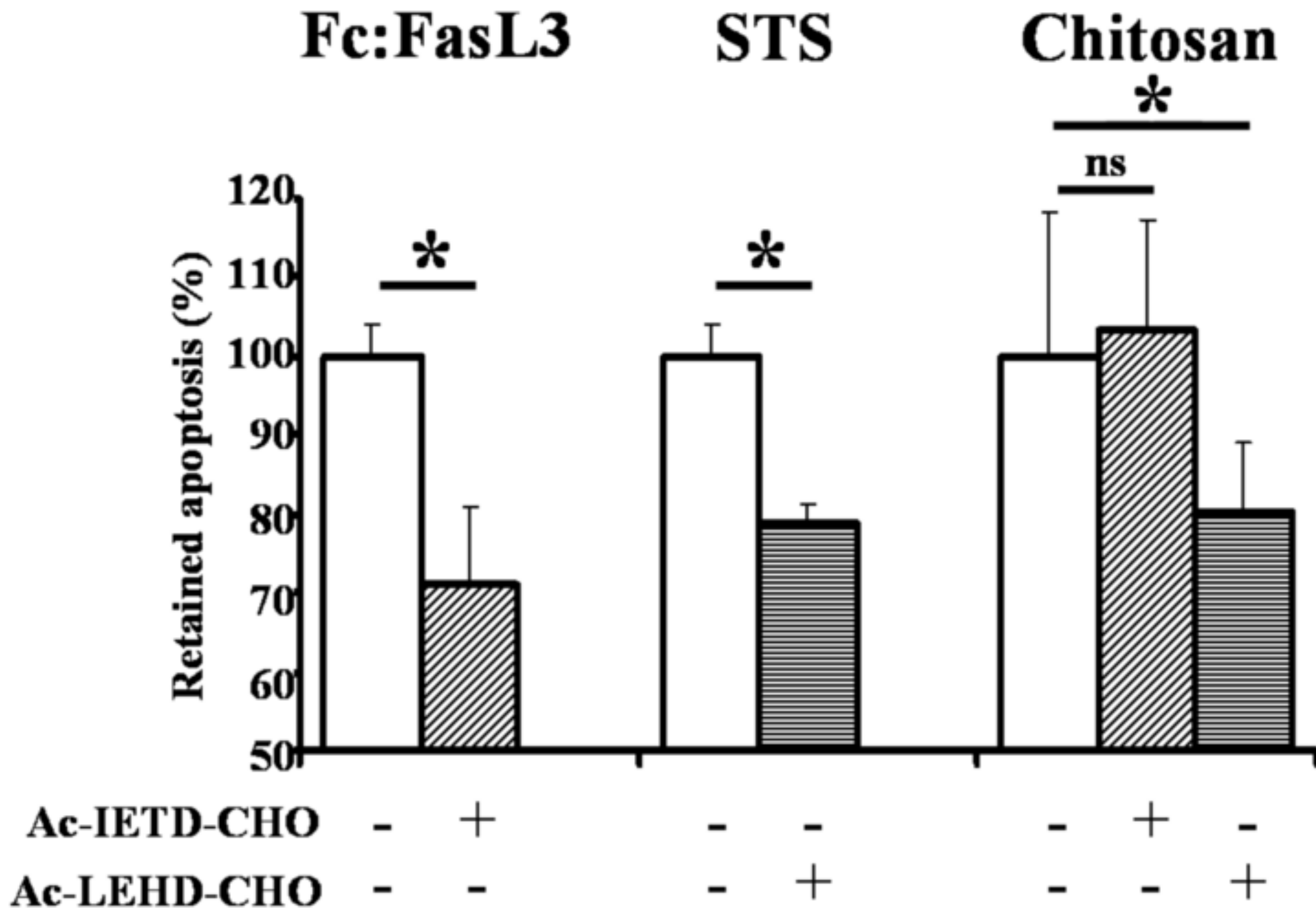

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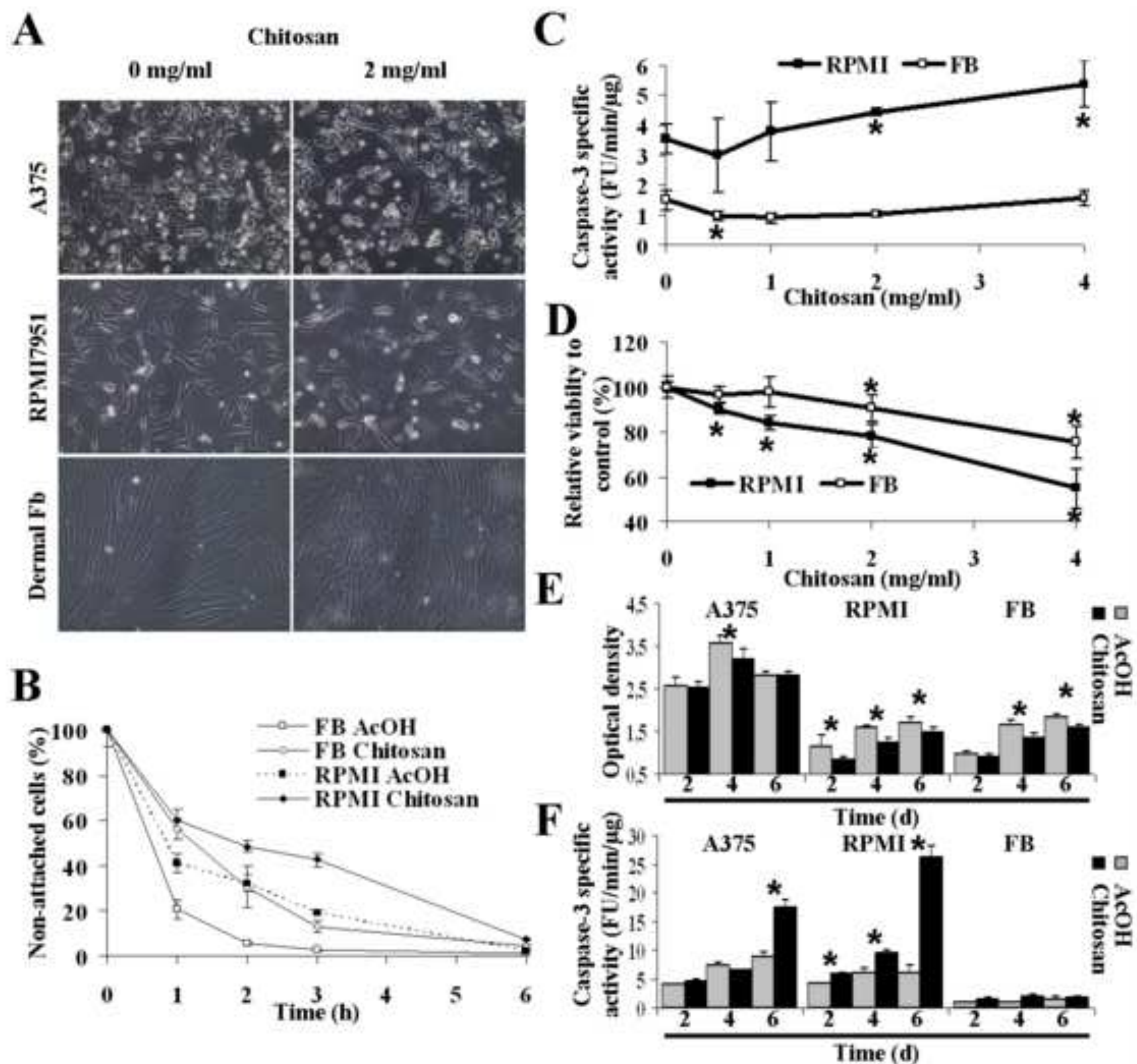

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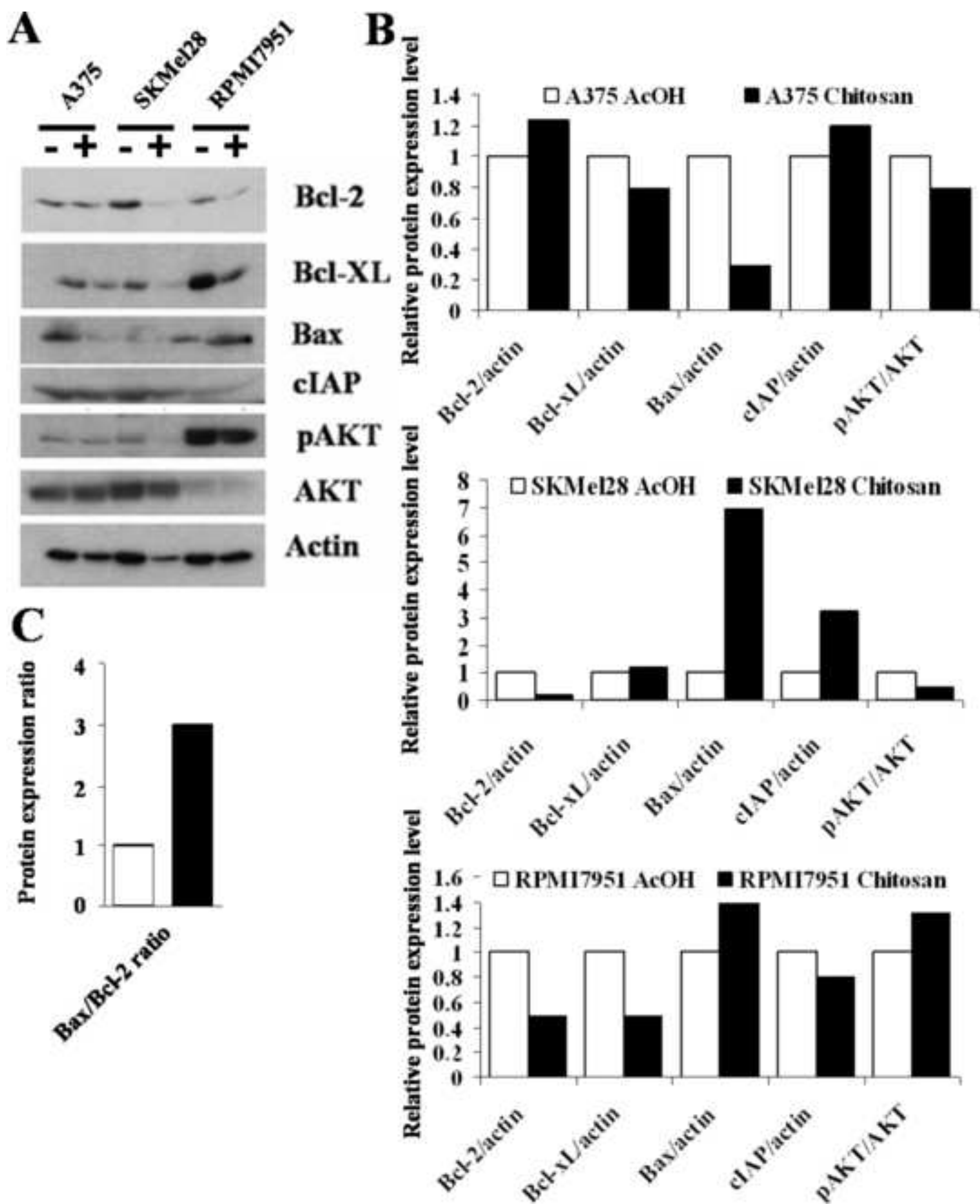

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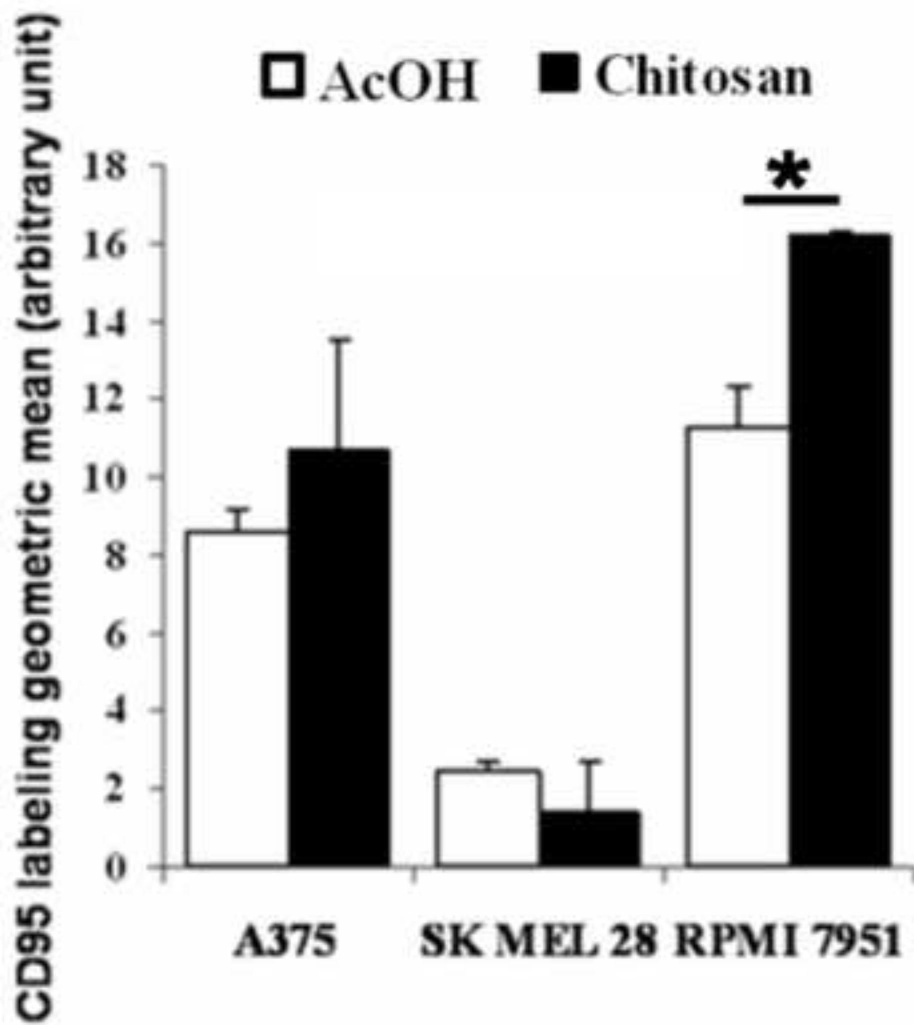

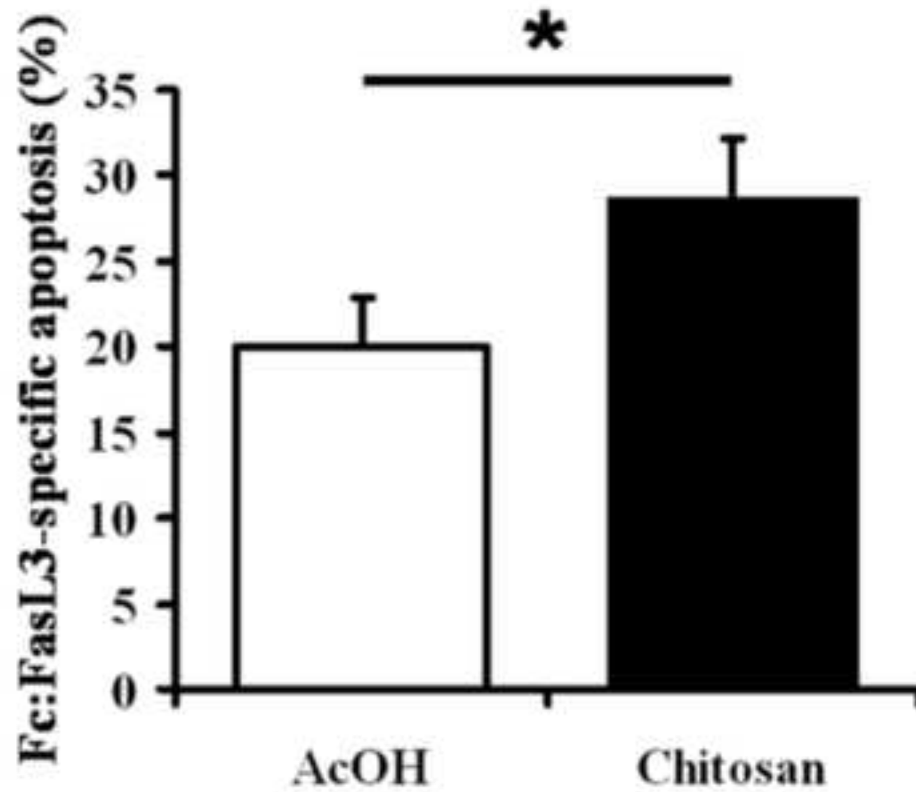

Figure 8
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