Surface acoustic waves cause net reduction in mouse and human melanoma growth in vitro

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Abstract

Low-energy surface acoustic waves (SAW) have been shown to be effective in curing urinary catheter infections and in enhancing neutrophil killing of bacteria. Here, we examined whether cellular immunity, the killing of melanoma tumor cells by T-cells, can be enhanced with the application of SAW. From the preliminary data, it appears that SAW is capable of reducing mouse and human melanoma cell growth independently of T-cell activity. In the B16 mouse melanoma model, growth in SAW-treated collagen/ fibrin gels was reduced by 27% at Day 2 and by 68% at Day 3 when compared to growth in control collagen/fibrin gels. A Propidium iodide assay, which fluoresces dead cells, suggested that the application of SAW killed B16 cells directly, since the SAW-treated gels showed almost twice the percentage of dead cells (44.25%) as compared to the control gels (26.09%). In addition, SAW-treated A175 cells, or human melanoma cells, showed approximately a 10% reduction in growth in collagen/fibrin gels in comparison to growth in control gels. T-cell killing and migration experiments performed with B16s in the presence or absence of SAW showed that SAW seems to adopt an independent mechanism in killing B16 melanoma cells, while enhancing T-cell chemotactic capacity. Our future experiments involving study of the effect of SAW on other types of dermatological cancers and understanding the underlying mechanisms of SAW in reducing tumor cell growth.

Key words: surface acoustic waves, melanoma, T-cells.

Introduction

Low energy surface acoustic waves (SAW) have been shown to reduce biofilm formation on urinary catheters in rabbits (Hazan et al., 2006). Recently, the FDA has approved SAW as a safe method to enhance wound healing (Howell-Taylor et al, 2008). Low-energy SAW devices generate and spread vibrational energy at frequencies of 100 to 300 kHz and amplitudes of 300 to 800 nm. These waves are propagated uniformly and in all directions, covering the entire system (Hazan et al., 2006). We hypothesized that the effectiveness of SAW was based on an underlying immunological mechanism since it showed clinical use in wound and bone healing, processes which involve immune cells. While there is no current research on the impact of SAW on immune cell function, recent unpublished studies in our lab have shown that SAW significantly improves the capacity of human neutrophils, which are innate immune cells, to kill bacteria within a three-dimensional in vitro fibrin gel environment. Based on these results, it was hypothesized that SAW enhanced bacterial killing in a tissue-like matrix by facilitating chemotaxis of neutrophils; the mechanical vibrations induced by SAW may cause activation of neutrophil through an unidentified mechanoreceptor. Since SAW appeared to enhance neutrophil function, we wanted to examine if other immune cells could also be stimulated by SAW. T-cells constitute an important arm of the adaptive immune system and are ubiquitously involved in defense against cancerous cells. We extended our study to examine whether SAW has an effect on T-cells ability to kill tumors cells.

Cutaneous melanoma is a type of skin cancer. Each year, the incidence of cutaneous melanoma continues to escalate, with over 50,000 new cases being diagnosed within the United States in one year. Approximately 10-20% of all such melanomas are located in the head and neck region and mortality is growing at a very high rate, second only to that of lung cancer. In most cases, excision of the tumor is the best treatment option available to patients (Rigel et al, 2000). The application of SAW can offer a treatment alternative perhaps for serious cases of melanoma in which lesions must be immediately excised, however, for other cases the SAW treatment may be used as an adjunctive therapy to enhance the effectiveness of surgery. The application of SAW to skin cancers may provide a treatment option for patients with metastatic melanoma, as SAW has been shown to directly kill melanoma cells. The present study was designed to examine if SAW can enhance the killing of melanoma cells by immune cells.

Methods

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but in cases where enhancement of T-cell cytolytic capabilities would cause a significant reduction in the tumor growth. SAW may also be useful in treating other significant dermatological cancers where lesions are superficial.

The current research asked whether SAW can induce apoptosis and/or antigen-specific cytolytic (CD8+) T-cells to kill mice melanoma cells in an in vitro collagen/fibrin gel model. T-cell-mediated killing of tumor cells is a multi-step process. T-cells must first migrate and find the tumor cells embedded in collagen/fibrin matrix. They must then recognize the melanoma cells bearing the appropriate antigen, SINIFEKl peptide in this case. T-cells can either induce apoptosis or directly kill the tumor cell by lysis (Cox et al., 2011). Cellular immuno-therapy, in which anti-tumor T-cells are infused in patients with melanoma, cures a very small percentage of them (Dudley et al., 2002, 2005; Morgan et al., 2006). A topical cream, like imiquimod, which activated immune cells through interaction with an innate receptor, has not been very successful in curing melanoma tumors either (Erickson et al., 2010). Instead of a chemical approach, we wanted to explore the outcomes of mechanical stimulation on function of immune cells to determine whether it is possible to enhance T-cell activation and response to melanoma.

We hypothesize that SAW can aid T-cell chemotaxis and therefore enhance killing of melanoma cells, just as it can aid neutrophil chemotaxis and enhance bacterial killing. However, surprising results emerged from the first experiment indicating that acoustic waves may not only affect lymphocytes but could directly impact the growth of melanoma cells. The presented research focuses on testing whether SAW can inhibit growth of mice and human melanoma cell lines independently of lymphocyte activity and examines possible underlying mechanisms.

Materials and Methods

Mouse OT-1 T-cells

CD8+ T-cells were isolated from the spleens of OT-1 mice and grown in a T-175 tissue-culture flask (Corning Inc.) with RPMI medium containing 10% fetal bovine serum (FBS) and 50 µM B-mercaptoethanol (BME). These OT-1 cells express a transgene encoding a T-cell receptor (TCR) that recognizes the ovalbumin peptide SINIFEKl in the context of mouse H-2k MHCI (Hoggquist et al., 1994).

SAW treatment

A battery-powered driver was connected to an "active element" made of a piezo ceramic plate that creates low-energy Surface Acoustic Waves. This element was attached to the 48-well plates and produces uniform acoustic waves that affect all the wells. The driver, specific for a 48-well plate, delivered 0.3 milliwatts per cm2 (Ha- zan, Zimmer et al., 2006). Since the administration of SAW can increase surface temperature (unpublished data) we added the cells in adjacent wells where there was no temperature increase (Figure 1).

Melanoma Cells

B16 melanoma cells derived from a primary tumor were used for the murine model. B16 is a well-characterized melanoma cell line. The A375 cells were derived from a primary tumor, used for the human model. The B16 cells were grown in T-75 tissue culture flasks with RPMI 1640 medium supplemented with 10% FBS and 50 µM B-mercaptoethanol and A375 cells were grown in DMEM medium supplemented with 10% FBS. Flasks were stored at 37°C in a 95% air/5% CO2 humidified atmosphere. Before an experiment, B16 cells were detached from the flask surface using 3 ml of 5% EDTA in PBS for 5 min and then neutralized with 7 ml PBS with Ca2+ and Mg2+.

A375 cells were detached using 2 ml 0.25% trypsin/5 mM EDTA in PBS for 5 min and then neutralized with 8 ml A375 media. Cells were then pelleted in a centrifuge at 1500 rpm (~50g) for 5-10 minutes, resuspended in their respective media. Cells were counted using a hemacytometer to determine the initial concentration and diluted in media to obtain the desired concentration for the experiment (107 cells/ml). In order to make B16 melanoma cell targets for the OT-1 T cells for the killing experiment, we pulsed, or incubated, B16 cells with SINIFEKl for 90 minutes before co-incubating them with OT-1 cells in the collagen/fibrin gels.

Melanoma Cell Growth in Fibrin/Collagen I gels

In order to simulate an in vivo environment, three-dimensional gels were formed in 48-well tissue culture plates using 100 µl of PBS containing 1 mg/ml human fibrinogen, 1mg/ml rat tail collagen I, 10% FBS, and 105 B16 or A375 cells. 5 µl of PBS containing 0.1 U thrombin was placed at the bottom of each well before adding the B16 cell mixture. Thrombin is a blood clotting enzyme that mediates the cleavage of fibrinogen to form a fibrin clot. Gels were allowed to clot for 15 minutes at 37°C in a 95% air/5% CO2 humidified atmosphere. 10 µl of PPACK (10-7 M) was added to inhibit thrombin and to prevent the formation of excessively hard gels. Each gel contains 0.1 mm colony in volume, and ~1500 µm in height. 1 ml of the respective cells at a concentration of 105 cells/ml were added to the B16 cell mixture immediately before individual gels were formed.

Fixing and Staining of B16 colonies

After 1 week, the 24, 48 and 72 hours plates were fixed with 1-2 ml of 3.7% formaldehyde for 15 minutes, then washed with PBS and stained with 2% methylene blue for 60-90 minutes. The methylene blue was then removed, 100 µl of Collagenase (2.5mg/ml) was added to each well, and plates were incubated for 30 minutes at 37°C in a 95% air/5%CO2 humidified atmosphere. 100 µl of Trypsin (2.5mg/ml) was added and plates were again incubated for 30 minutes. Once the gels had liquefied, 700 µl of 5M EDTA was added to each well and plates were incubated for another 5 minutes to disaggregate the cells. For gels with a 105 concentration, two 1:10 serial dilutions in PBS with Ca2+ and Mg2+ were made. 100 µl of the final dilution was transferred into a well in a 6-well plate, containing 1.5 ml of respective media, 6-well plates were incubated at 37°C in a 95% air/5% CO2 humidified atmosphere for 1 week to allow colony formation. Plating efficiency of B16 is ~60% and ~40% for A375s.

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Lysing and Plating Fibrin/Collagen I gels

Gels were lysed to harvest cells at intervals of 24, 48 and 72 hours. The overlaid media was removed, 100 µl of Collagenase (2.5mg/ml) was added to each well, and plates were incubated for 30 minutes at 37°C in a 95% air/5%CO2 humidified atmosphere. 100 µl of Trypsin (2.5mg/ml) was added and plates were again incubated for 30 minutes. Once the gels had liquefied, 700 µl of 5M EDTA was added to each well and plates were incubated for another 5 minutes to disaggregate the cells. For gels with a 105 concentration, two 1:10 serial dilutions in PBS with Ca2+ and Mg2+ were made. 100 µl of the final dilution was transferred into a well in a 6-well plate, containing 1.5 ml of respective media, 6-well plates were incubated at 37°C in a 95% air/5%CO2 humidified atmosphere for 1 week to allow colony formation. Plating efficiency of B16 is ~60% and ~40% for A375s.

Figure 2. B16 growth in Fibrin/Collagen I gels (N=3)

In the indicated times (1 day = 12 hours of SAW treatment), the gels were harvested, and B16 cell content was determined by a clonogenic assay.

Figure 2. B16 growth in Fibrin/Collagen I gels (N=3)
with SAW treatment. The preliminary experiment below indicates that acoustic waves alone kill 11% of the B16 cells (net growth is reduced from 100% to 89%, Figure 5). T-cells alone kill 57% of the B16 cells (since 43% of the cells survive when just exposed to T-cells and not SAW). With both acoustic waves and T-cells killing is enhanced to 72%, since only 28% of the cells survive.

T-cell migration across collagen/fibrin gels is enhanced by SAW treatment. We hypothesized that enhancement in T-cell mediated killing might be due to increased migration into collagen/fibrin gels in response to SAW treatment. We plotted T-cells on uncoated porous chambers and observed their migration through the pores into a lower compartment in response to known chemoattractants, such as IP-10 and RANTES. In both cases, T-cell migration in SAW-treated wells was higher than in the control wells (Figure 6).

Discussion

Previous work in the lab has primarily tested low-energy surface acoustic waves on neutrophils’ ability to kill bacteria. The effects of SAW on T-cells’ cytolytic ability and on the tumor cells themselves are two largely unexplored areas. This paper presents the data from preliminary experiments in which tumor cell growth and killing were studied. There is a strong indication that SAW is able to dramatically inhibit tumor growth directly without the presence of immune cells (Figures 2, 3). The experiments conducted in the murine model, using B16 cells, have shown that when B16 cells are grown in a tissue-like environment are subjected to 12, 24, and 36 hours of SAW treatment, there is a significant reduction in tumor cell growth. The effect is most pronounced after 36 hours of SAW treatment, when there is approximately a 68% reduction in B16 cell growth (Figure 2), indicating that the longer SAW was applied, the more B16 cell growth was inhibited. It remains to be investigated if SAW reduces B16 count by directly killing these cells or just slowing down tumor cell proliferation. We measured the temperature of the wells in the tissue culture plate during application of SAW and found that the four wells directly above the element had slightly elevated temperature, while the remaining wells did not show an increase in temperature, irrespective of the distance from the SAW element (data not shown). Since we did not use the wells directly above the SAW element in any of the experiments (Figure 1a), we can conclude that increased temperature is not the mechanism of cell death.

We were curious to understand the underlying mechanism employed by SAW in reducing mouse melanoma cell growth. Preliminary experiments indicate that the application of SAW significantly reduces B16 melanoma cell growth in collagen/fibrin gels (Figure 2). The two plates contained the same initial number of cells and gels were formed in the exact same conditions, therefore it can be concluded that the growth inhibition is due to SAW only. B16 cells in the absence of SAW grow very efficiently in these gels (Figure 2, Control). When SAW was applied, there was approximately 27% reduction in B16 cell growth at Day 2 and approximately 68% reduction at Day 3.

SAW treatment induces death of murine melanoma cells. SAW administration lead to an increase in the number of Propidium Iodide positive B16 cells after 48-hours (Figure 4). In order to understand the underlying mechanism of reduced cell growth, we tried to determine whether SAW was killing B16 cells or just slowing down tumor cell proliferation. We used propidium iodide (fluorescent dye that only binds to dead cells) to assess the number of dead B16 cells in our gels after incubation with SAW. This would tell us whether SAW acts to directly kill B16 cells in these gels. SAW-treated gels contained almost twice the percentage of dead cells (44.25%) as compared with the control gels (26.09%). This shows that SAW reduces B16 growth by inducing cell death, but does not rule out growth inhibition as a mechanism of controlling tumor cell growth.

SAW inhibits human melanoma cell growth. We wanted to test whether the effects of SAW on murine melanoma cells could be reproduced in human melanoma cells. A375 is a human melanoma cell line that was originally derived from a primary tumor. It has the ability to form colonies just like B16 cells which enables us to use the clonogenic assay (described in Methods) to assess the growth of these cells in collagen/fibrin gels. Our preliminary studies show that although A375 growth does not appear to be significantly affected at Day 1—after 12 hours of SAW treatment—there is a significant reduction (-90%) in growth at Day 2 (Figure 5).

T-cell-mediated killing of B16 melanoma is enhanced when SAW is applied. The preliminary experiment below indicates that acoustic waves alone kill 11% of the B16 cells (net growth is reduced from 100% to 89%, Figure 5). T-cells alone kill 57% of the B16 cells (since 43% of the cells survive when just exposed to T-cells and not SAW). With both acoustic waves and T-cells killing is enhanced to 72%, since only 28% of the cells survive.
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The effects of SAW on melanoma cell growth were examined using a Propidium Iodide assay. T-cells suspended in RPMI medium containing 0.1% BSA were added to wells on 24-well plates. Melanoma cells were harvested from gels with and without SAW at the 48-hour time point (24 hrs of SAW treatment). We observed that gels treated with SAW not only had approximately twice the percentage of dead cells (Figure A) but also showed a significant reduction in cell number as determined by statistical analysis. It is possible that SAW is capable of inhibiting tumor cell division or inducing apoptosis. For future analysis, an apoptosis assay will also be used to better understand the underlying mechanism of SAW's anti-tumor effects.

We also tested the applicability of our model to human melanoma cells. The A375 cell line, derived from a human melanoma tumor, was used to perform the growth inhibition experiment with and without SAW. Based on the results, it appears that SAW has a similar effect to that seen in B16 cells; that is, at the 48 hour time point only, SAW-treated gels contained a significantly lower number of A375 cells than control gels (Figure 3).

Lastly, we tested the effect of SAW on murine T-cells' killing capability. Since we wanted to examine the effect of SAW and T-cell killing independently, we had four different conditions: a) B16 only; b) B16 + OT-1; c) B16 + SAW; and d) B16 + OT-1 + SAW. By comparing B16 growth in 24 hours for conditions a) and b), we measured T-cell cytolytic ability. By comparing growth in conditions a) and c) we measured SAW's ability to kill/inhibit growth. And by comparing a) and d) we determined how much killing could occur in the presence of both T-cells and SAW. We found that gels subjected to both T-cells and SAW exhibited the maximum killing, suggesting that SAW could be used as a supplementary tumor killing mechanism to T-cell mediated immuno-therapy. It seems likely that the effect of T-cells and SAW on B16 killing is additive and each employs a separate mechanism to reduce tumor cell growth. However, SAW appears to enhance the capacity of T-cell to migrate in response to chemotactic factors, perhaps by allowing more of the chemoattractant to be diffused among the cells or by triggering T-cell activation.

Applying SAW to human melanoma in a clinical situation may not be a fully viable option, since most melanoma lesions are surgically removed. Yet, our studies indicate that SAW might have therapeutic value in other superficial dermatological tumors, where excision is not preferred. We plan to examine the effects of SAW on human squamous cell and basal cell carcinomas, skin cancers in which lesions are often small and numerous and surgery is not always practical. We will observe the effects of SAW on cell growth both in vitro, using cell lines derived from human tumors, and eventually in vivo, using SHH-1 hairless mice that naturally present basal carcinoma lesions.

Furthermore, we will examine whether SAW enhances the sensitivity of tumors to chemotherapeutic agents, such as topical imiquimod, which is used clinically for patients with squamous cell carcinoma (Neubert et al, 2008). Finally, if SAW does enhance melanoma killing, we may test whether it also promotes killing of virally infected cells.

In conclusion, this research project, which suggests that SAW affects growth and killing of tumor cells, and the studies proposed above, will determine whether application of SAW to solid tumors will be beneficial in treating cancer.

References


