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Regulation of Id-1 Gene Expression in Aggressive Breast Cancer and Glioblastoma Cells Using Cannabinoid Compounds

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Abstract

We investigated Id-1 gene expression in aggressive breast cancer cells and glioblastoma cells, and compared Id-1 expression in control cells with cells treated with one of the cannabinoid compounds, cannabidiol (CBD) or tetrahydrocannabinol (THC). The tumor cells were treated with CBD or THC, and the control group was treated with ethanol. After 2-3 days of treatment, cells were harvested and proteins extracted from them. Following protein electrophoresis, Western blot technique was used to analyse the expression of Id-1 protein derived from the samples. Using a rabbit monoclonal anti-human Id-1 antibody, and a goat anti-rabbit HRP as a secondary antibody, the intensities of the bands produced following film exposure were used to compare drug-treated cells with controls. Id-1 gene expression was decreased in tumor cells treated with cannabinoid compounds. We also aimed to visualize tumor cell migration through a living tissue and to determine the possible inhibition of migration upon treatment with CBD. Tumor cell migration through a living tissue was studied by placing GFP-labelled glioblastoma cells on top of Sprague-Dawley rat brain slices. Migration was studied using the Carl-Zeis cell monitor. Glioblastoma cells placed on brain slices of Sprague-Dawley rats were found to migrate through the tissue to the other side of the slice. The morphology of glioblastoma cells was also found to be modified upon treatment (changes in cell-to-cell contacts). This study shows that Id-1 downregulation strongly correlates with a reduced tumor aggressiveness and that Id-1 might represent a potent therapeutic target for breast cancer and glioblastoma.

Keywords: breast cancer; glioblastoma; gene expression; Id-1 gene.

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1. Introduction

Carcinogenesis requires several genetic and morphological changes in the cells leading to loss of cellular differentiation, cellular proliferation, invasion and metastases. Metastases represent the final steps and underscore fatality in affected persons [1]. Breast cancer and glioblastoma are difficult to cure particularly when they have undergone invasion.

The inhibitor of DNA binding-1 (Id-1) encodes a helix-loop-helix (HLH) protein that has no DNA binding ability and thus can inhibit DNA binding and transcriptional activity of other HLH proteins with which it interacts [2]. It has roles in cell differentiation and proliferation and is expressed mostly during early development (1). It is potentially important for angiogenesis and neurogenesis. Reducing Id-1 using antisense technology significantly reduced breast cancer cell proliferation and invasion in culture [3]. Reducing Id-1 over-expression might therefore be a potential therapeutic strategy for the treatment of metastatic cancers.

Cannabinoids were discovered as potential candidates to downregulate Id-1 expression in metastatic cancers. Several of these compounds are agonists of two G protein-coupled receptors, CB_1 and CB_2 [4]. These receptors have tumor-inhibiting ability [5]. While THC has significant CB_1 and CB_2 activity, it is also psychotropic thus limiting its use. However, CBD has low CB_1/CB_2 activity and it is not psychotropic [6], potentially making it a very important agent in tumor management.

This study aimed to investigate Id-1 expression in aggressive breast cancer cells and glioblastoma cells and to compare Id-1 expression in control-treated tumor cells versus CBD and THC-treated cells. It also aimed to visualize tumor cell migration through a living tissue and to determine the possible inhibition of migration upon treatment with CBD.

Animal use in this study conformed with approved standards as outlined by the Institutional Animal Care and Use Committee [7]

2. Materials and Methods

I. *Id-1 expression in aggressive breast cancer and glioblastoma cells*

Cell culture and treatments

The human breast cancer cell line MDA-MB231 and glioblastoma cell line U251 obtained from the American Type Culture Collection were used. Cells were cultured in 75cm^3 plates with 10% FBS. When the cells became confluent, they were harvested for sub-cultures. 2mls of trypsin were added to each plate and allowed for about 3 minutes in the incubator. 12mls of 10% FBS added and the solution transferred to 50ml tubes and centrifuged. Supernatant was discarded in each case. 10mls of 10% FBS was added and the cells were dispersed in the solution. Cell counting was done with the help of the haemocytometer. 150,000 MDA-MB231 cells were plated out in new 75cm^3 and 120,000 for U251 cells. After a full day of culture, cell treatment started. Medium with 10% FBS was aspirated and different concentrations of cannabidiol and tetrahydocannabinol in 0.1%FBS

introduced for treatment. The control plates were treated with ethanol. Media were replaced every day and cells harvested on 2nd and 3rd days of treatment using trypsin.

Protein extraction and assay

Protein extraction was done using M-PER Mammalian Protein Extraction Reagent by ThermoScientific with 1% protease inhibitor, 1% phosphatase inhibitor and 1% EDTA. After addition of this reagent, cells were spun for 30 mins at high speed in the cold room. Supernatant was immediately decanted and stored in the freezer. Assay was done with using the BCA reagent by ThermoScientific. BCA reagents were added to appropriate volumes of proteins in 96-well plates. Incubation was done at 37^0C for 30mins and absorbence measured with a spectrophotometer at 562nm wavelength. Protein concentrations were determined by comparing the samples with standards.

Western blotting

Proteins were separated by SDS-PAGE, blotted onto a membrane and probed with an anti-Id-1 antibody. The primary antibody used was a rabbit monoclonal anti-human Id-1 from Biocheck Inc. The secondary antibody was a goat anti-rabbit HRP. Following treatment with femto, band intensity was obtained from radiographs and analyzed.

II. ***Tissue slice assay experiments***

Conditioned media

Mouse fibroblasts were taken from the freezer, thawed and plated out in tissue culture plates with 10% FBS media. Cells were cultured at 37^oC in the incubator until confluency. 10% FBS media were removed and replaced with plain RPMI media supplemented with 5ug/ml insulin. Conditioned media were collected 3 days later. The collected media were pooled together and divided into 10ml aliquots for use.

GFP-U251 cells

After thawing, 10% FBS was added and centrifugation done. Pellets were resuspended in fresh 10% FBS and incubated at 37^oC. After a few days, cell confluency was achieved. 10% FBS was aspirated and flask washed with plain RPMI media. Trypsin was added and reaction stopped with 10% FBS after 3 minutes. Cell count was done and cells were re-suspended and spun for 5mins. The supernatant was discarded. Cell pellet was re-suspended in plain RPMI media and spun a second time. Supernatant was discarded. Pellet suspended in calculated amount of plain RPMI media to give a concentration of 10,000 – 50,000 cells/ul.

Rat brains and procedure

Pregnant Sprague-Dawley rats ordered from a vendor were monitored till they had their pups. Just before the experiment, the 1-3 day old pups were euthanized and their brains harvested in sterile PBS. 500um thick rat

brain slices were prepared using razors and slicing blocks. The slices were suspended in neurobasal media until ready for use. 2mls of RPMI or plain neurobasal media were added in the wells. One brain slice was placed on each of the 8um cell inserts, covered with 150ul of neurobasal media and suspended on the well plates containing RPMI or neurobasal media. 1ul of suspended GFP-U251 cells carefully placed on each slice and incubated for 30 minutes. After 30 minutes, the inserts containing the tissue slices were then placed in new 12-well plates containing 1.2mls of conditioned media and incubated for 30 minutes at 37^{o}C. I.5uM CBD were introduced into half of the wells while the other half were control wells. GFP cells were visualized under green filter to confirm they were on top of the brain slices and incubated for 3 days at 37^{o}C. After 3 days of incubation, plates were taken out of the incubator and conditioned media aspirated. 1ml glutaraldehyde was put into each well to fix the migrating cells. After 15 minutes incubation at room temperature, glutaraldehyde was aspirated and the inside of the cell insert was wiped with cotton bud to remove any U251 cells trapped on top. 1ml of PBS was added to each well and viewed under green filter microscope. After microscopy, staining with crystal violet was done and incubated for 2 hours at room temperature. The underside of the cell insert was checked again.

Timed assay study

GFP-labelled U251 cells were placed on brain tissue slices on cell inserts. These inserts were placed on well plates and cell migration through the brain slices monitored using the Carl-Zeis cell observer. Serial pictures were taken to follow cell migration through the brain tissue slice. The Carl-Zeis cell observer is a computerized time-lapse vidoe microscopy system used to monitor cell fate and movement. This system enables monitoring of live cells in vitro and quantification of their motility over hours or days. It consists of a microscope, digital camera, an incubator with a computer where a software program drives the microscope stage.

3. Results

I. Id-1 expression in glioblastoma and breast cancer cells

After treatment of U251 cells with cannabinoids, pictures of the cells were taken after 2 or 3 days of treatment, and compared with the picture of the control-treated cells. Harvesting of the treated and control cell groups was then performed. Medium used for cell harvesting was M-PER Mammalian Protein Extraction Reagent by ThermoScientific with 1% protease inhibitor, 1% phosphatase inhibitor and 1% EDTA. Protein assay was done using BCA reagent and concentrations of the proteins in control and treated groups calculated using a spectrophotometer. Gel electrophoresis and transfer of gel electrophoresis products to the membrane was done. The membrane was incubated with non-fat milk to block non-specific binding. Primary antibody incubation was done using a rabbit monoclonal anti-human Id-1 antibody from Biocheck Inc. Secondary antibody incubation was performed using a goat anti-rabbit HRP. Femto was added and films taken and developed in the dark room. The results are shown in figures 1-4. Id-1 protein expression was downregulated by CBD in cancer cells.

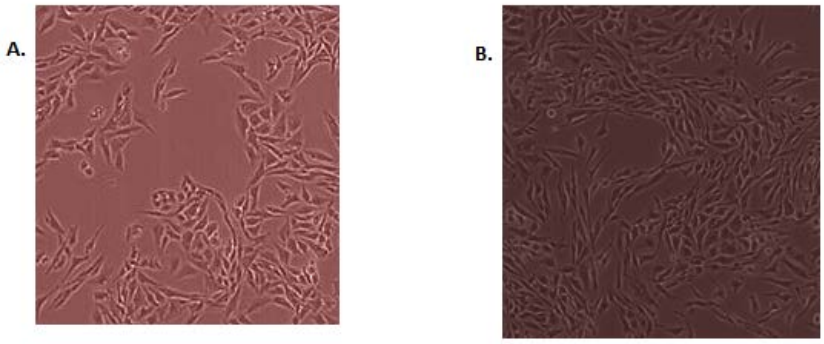

Figure1: Control-treated U251 cells taken on (A) 2nd day of treatment and (B) 3rd day of treatment.

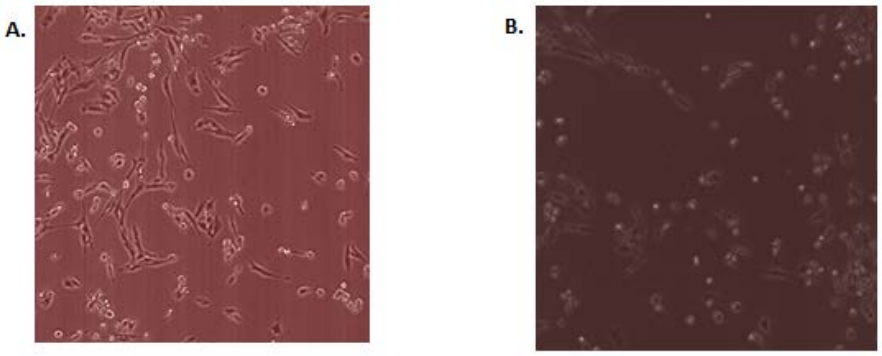

Figure 2: CBD-treated U251 cells taken on (A) 2nd day of treatment and (B) 3rd day of treatment.

Figure 3: Id-1 blots of U251 cells following a 2-day treatment with vehicle control or CBD. Panel A shows the Id-1 blot and B shows the actin blot.

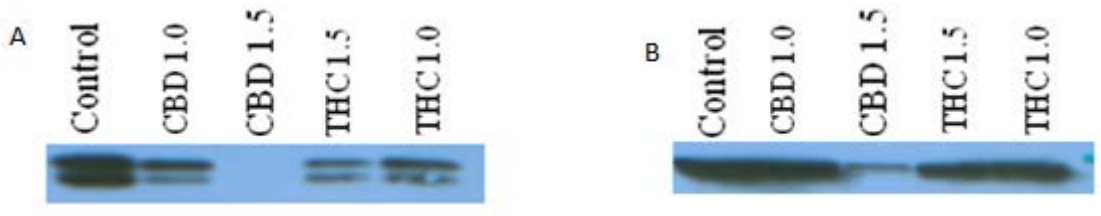

Figure 4: Id-1 blots of MDA-MB231 cells following a 3-day treatment with vehicle control, CBD or THC. Panel A shows the Id-1 blot and B shows the actin blot.

II. Results of timed tissue slice assay experiments

After deposition of GFP-labelled U251 cells on brain slices from 1- 3 day old Sprague-Dawley rats, the cells were tracked under the Carl-Zeiss computerized video microscopy. Cell migration was monitored by observing the relative fluorescence of the GFP cells on both the top and at the other side of the slice. Initially, the fluorescence was strong on the top where the deposit was made but over time, it faded and fluorescence increased on the other side of the slice as the cells made their way through the slice. The results are shown in figures 5 and 6.

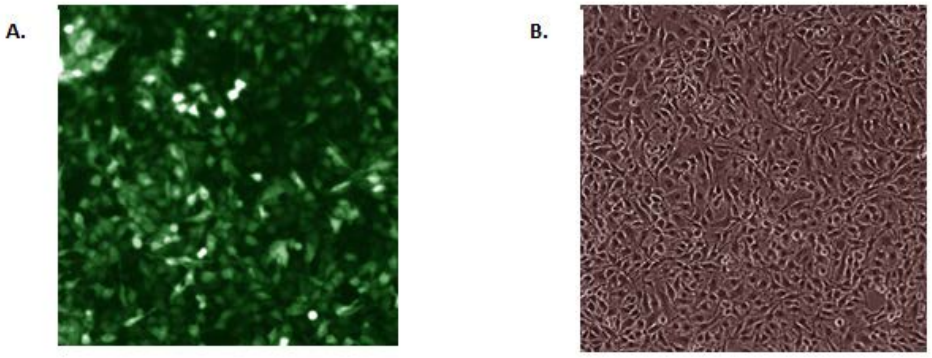

Figure 5: GFP-labelled U251 cells on Sprague-Dawley brain slices. Picture A was taken using the green filter and B taken using the light phase.

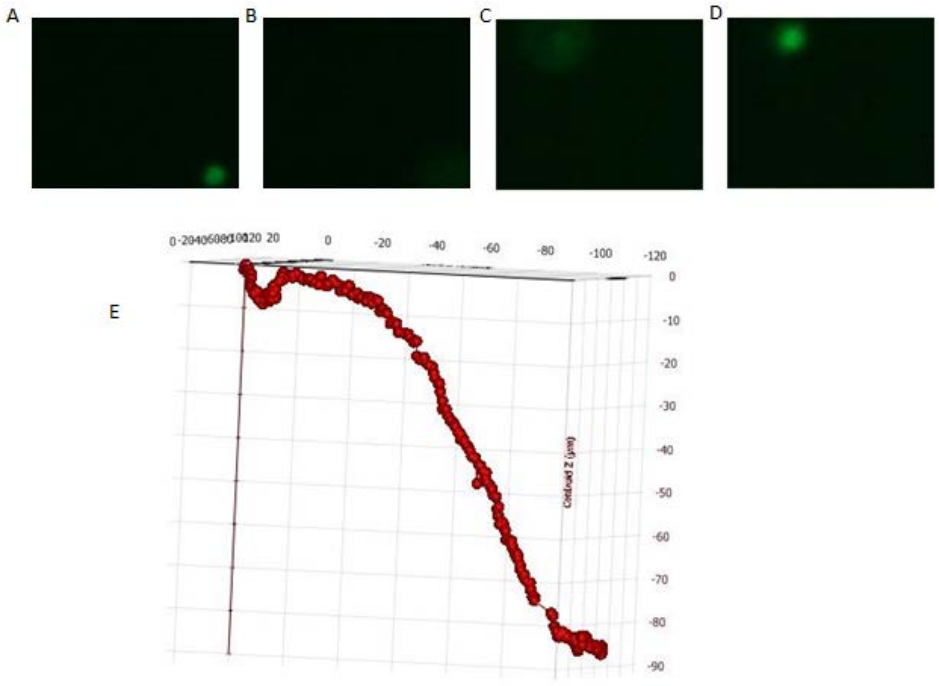

Figure 6: Tracking of GFP-labelled U251 cells on Sprague-Dawley rat brain slices. A corresponds to the top of the slice at start; B to the bottom of the slice at start; C to the top of the slice at end; D to the bottom of the slice at end; E represents the track of the GFP-labelled cell through the brain slice.

4. Discussion

I. Id-1 expression in glioblastoma and breast cancer

This study examined Id-1 gene expression in glioblastoma and aggressive breat cancer cells and compared results obtained from CBD and THC-treated cells with vehicle control-treated cells. Figure 1 shows the characteristic morphology of U251 cells on slides (i.e. spindle-like shape). There is evidently an increase in cell number when compared slides A and B obtained on day 2 and 3 of treatment with ethanol. The morphology of U251 cells treated with CBD is shown in Figure 2. Cells appear more rounded. This probably explains the reduction in cell proliferation and migration already noted in CBD-treated cancer cells [8]. Figures 3 and 4 correspond to Western blots of Id-1 protein obtained from glioblastoma and breast cancer cells respectively. These results show a downregulation in Id-1 expression in cells treated with CBD and THC in comparison to control-treated cells. A dose-dependent downregulation was consistently found in this study. This confirms the potential importance of Id-1 in tumor progression. Previous studies by McAllister and his colleagues [9] had demonstrated a downregulation in Id-1 promoter activity when CBD-treated breast cancer cells were compared with control-treated cells. Our findings are in agreement with earlier findings showing that targeting Id-1 expression, even partially, in breast cancer cells reduced invasion in vitro and breast cancer metastasis in preclinical animal models.

II. Timed tissue slice assay experiments

This study aimed to visualize migrating tumor cells as they made their way through living tissue and also aimed to investigate for possible inhibition of tumor cell migration by cannabinoids. Previous studies using Boyden chambers had shown a reduction in breast cancer cell migration across a reconstituted basement membrane following treatment with CBD and THC [9]. CBD was found to be the most potent inhibitor of invasion. In this experimental model, GFP-labelled U251 glioblastoma cells were placed on brain tissue slices prepared from 1-3 day old Sprague-Dawley rats. These cells were monitored using the Carl-Zeiss microscope and the results are shown in Figures 5 and 6. U251 cells were tracked by relative loss or gain of fluorescence on both sides of the brain slice. As the cells made their way through the slice, the fluorescence on the top side where the cells were initially deposited was reduced and the fluorescence on the reverse side was increased. U251 cells migrated through the brain slice and reached the reverse side of the brain slice. It was difficult at the time of the experiment to demonstrate a potential inhibition of cell migration in the CBD-treated brain slices and compare the results with controls. The results obtained from CBD-treated brain slices were not interpretable.

5. Conclusion

As the search for potent anti-cancer agents continues, one of the main considerations in improving treatment is toxicity. Id-1 expression is very high in aggressive cancer cells but low in normal tissues. This could potentially eliminate unwanted toxicities associated with many current treatments. Cannabinoid agonists working through CB_1 and CB_2 receptors have been shown to act as tumor inhibitors in a variety of cancer models. Present evidence in this study demonstrates a decrease in the expression of the pro-metastatic Id-1 gene in breast cancer

and glioblastoma cells treated with CBD (and to a lesser extent treated with THC). Thus, a rational drug design strategy using these agents could potentially be used for the management of aggressive breast cancer and glioblastoma and, possibly, other cancer types.

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