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Abstract

Inositol phosphates are naturally occurring compounds that regulate diverse cellular processes including apoptosis. Apoptosis is a mechanism by which cells undergo natural death to maintain cellular homeostasis. It causes cell death in areas during a state that is harmful to the body. It also regulates cellular development. Previous work has shown that exogenously administered, as well as endogenously manipulated inositol phosphates bring about apoptotic changes. It has been demonstrated that cellular levels of inositol phosphates, particularly higher inositol phosphates such as inositol hexakis-phosphate (IP_6) and diphosphoinositol pentakis-phosphate (IP_7) levels increase during apoptotic conditions. In this study, we have attempted to separate and identify higher inositol phosphates such as IP_6 by polyacrylamide gel electrophoresis (PAGE) and shown that changes in inositol phosphate levels can be detected by this method. Cells were treated with etoposide to induce apoptosis, and apoptotic cells were observed under UV light following ethidium bromide/acridine orange staining. This staining showed that IP_3 - IP_6 induced apoptosis in SW480 cells with IP_6 being the most effective inducing agent. The extracts from apoptotic and control cells were then loaded onto the polyacrylamide gel and run along with standard IP_6. Results showed that IP_6 could be detected using the PAGE method and that cellular levels of IP_6 were increased in SW480 cells, in which apoptosis had been induced by etoposide. Our results demonstrated that this technique could be utilized instead of the laborious radioactive labeling and HPLC separation method to study the changes in cellular levels of inositol phosphates particularly IP_6.

Introduction

Cancer research over the past decade has been a growing field. It is necessary to promote longevity for those in need of cancer treatments. Cancer treatments begin their journey in a lab, as researchers experiment with cells using different methods to find cures. Firstly, it is important to understand how normal and abnormal cells differ. A normal cell in the human body reproduces through regulated signals, and when cultured, or grown in the lab for research, they stop dividing once they carpet the bottom of the flask, a process known as contact inhibition. They are also characterized as having a function. Tumor cells on the other hand, do not stop dividing even when they cover the bottom of a flask, i.e. they do not exhibit contact inhibition. Instead, they continue to grow, and it can be assumed that they do not posses signaling cells like normal cells. They also do not appear the same as normal cells, and since structure corresponds with function, if the shape has been modified compared to that of a normal cell, one can assumes that the cell is abnormal, and is harmful to the body. Normal cells perform specific functions in the body, however, abnormal cells do not have special functions to perform, and therefore they lack normal shape. The majority of cancer related deaths are due to the spread of cancer cells from their primary site to other parts of the body. Treatments aimed at specifically inducing apoptosis in cancerous cells should greatly reduce the number of cancer related deaths. Ideally, to see if the treatments applied to the cells have any effect i.e. the cells should reach a high percentage of apoptosis. Apoptosis is a technical term for cell suicide. In apoptosis "the cell surface is altered, displaying properties that cause the dying cell to be rapidly phagocytosed, either by a neighboring cell or by a macrophage" (Alberts et. al. 2002). The use of signaling mechanisms with metabolic intermediates such as inositol phosphates can help increase the amount of apoptosis. We have previously shown that extracellular administration of IP_4, IP_5, and IP_6 increased apoptosis in a dose-dependent manner (Agarwal et al. 2009).

Inositol phosphates are a group of six-carbon

carbohydrates that are phosphorylated and found in nature and have important roles in intracellular communication (Turner et al 2002). In mammalian cells, they play important signaling roles in regulating diverse cellular processes including vesicular trafficking and apoptosis (Berridge et. al 1989). Previous experiments have shown that IP $_{4-6}$ greatly impact apoptotic behavior. The same is true of the common chemotherapy drug etoposide. Etoposide is a chemotherapeutic medicine that can be administered orally or injected. Etoposide is an inhibitor of the enzyme Topoisomerase II and is used as a form of chemotherapy in combination with other drugs. It breaks the genetic material inside the cancer cells and prevents them from further increasing in number, and then eventually the cells die.

Analysis of inositol phosphates has suffered greatly from the lack of simplified detection techniques. Although, the high-performance liquid chromatographic (HPLC) method has allowed mass analysis of inositol phosphates from cells and tissues (Guse et al 1995), this method is still insensitive, laborious and gives an under-estimation of the inositol phosphates. Other methods use radioactive precursors to label metabolically active cells and tissues in order to detect higher inositol phosphates, but the use of radioactivity is hazardous and also expensive. Therefore, Saiardi and colleagues developed a new method of detection of inositol phosphates. The rapid and simple method for the analysis makes use of polyacrylamide gel electrophoresis (PAGE) with toluidine blue staining in order to visualize IP_6 and DAPI staining for IP_7. Although Saiardi and colleagues originally used standard inositol phosphates for their research, their results suggest that this method can be used to determine levels of inositol phosphates under cellular processes such as apoptosis. Therefore, in this manuscript, we report the use of this PAGE method to examine whether levels of IP_6 in SW480 colorectal cancer cells change following induction of apoptosis by etoposide.

Materials and Methods

Cell Culture

Using standard sterile techniques, SW480 colorectal cancer cells were maintained in flasks containing 12ml DMEM media with 10 % fetal bovine serum (FBS) at 37^{o}C. Once cells reached confluency, they were plated in 6-well plates at a density of 5 x 10^4 cells per well and incubated at 37^{0}C for 24 h. The media was then replaced with starvation media (DMEM with 2% FBS) and cells left in the incubator at 37^{0}C for 24 hours.

Induction of Apoptosis

Apoptosis was induced by adding etoposide [100nM] or inositol phosphates (IP_{3-6}) [100µM] in experimental samples. DMSO and H_2O were used as vehicle controls for etoposide and inositol phosphates respectively. The cells were then incubated overnight. Media were removed from the cells and the cells were washed with 1X PBS, pH 7.4. The cells were either lysed with lysis buffer (Agarwal et al 2009) for electrophoresis or stained with acridine orange and ethidium bromide for visualization under microscope and determination of the extent of apoptosis.

Acridine Orange/Ethidium Bromide Staining

Cells were washed with 1X PBS and then stained with a solution containing equal volumes of 100 mg/ml acridine orange and 100 mg/ml ethidium bromide. Cells were observed under U.V light using Nikon fluorescence microscope fitted with a digital camera. Live (green) and apoptotic (orange) cells were counted (Agarwal et. al 2009) for determining the percent apoptosis. Approximately 200-300 cells were counted for each treatment. Results are expressed as means ±SE from three independent experiments. Student's "t" test and one way ANOVA was to determine significance. Statistical significance was defined as

Preparing Cell Extract

The cells were washed with 1X PBS after the media was removed. 100µl of lysis buffer was added to culture dishes to extract total inositol phosphates. The cells were scrubbed and left on ice for 15min. The solution was then transferred to 2ml Eppendorf tubes and centrifuged at 4^{o}C, 18,000xg for 10 min. The supernatant was collected and used for gel electrophoresis.

Polyacrylamide Gel Electrophoresis

The samples were run on a 33% polyacrylamide mini gel (7.925 ml of 40% Acryl/Bis 19:1, 0.95ml of 10X TBE, 0.55ml of dH_2O, 67.5µl of 10% APS, 7.5 µl of TEMED), for almost 3 hours or until the dye front reached 2/3rd of the gel length. The samples were mixed with Blue/Orange Loading 6X Dye [0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll 400, 10mM Tris-HCl (pH 7.5) and 50mM EDTA (pH 8.0)] prior to loading onto the gel (Saiardi et. al 2009).

Toluidine Blue Staining

The gel was agitated for 30 min in a filtered staining solution (20% methanol, 2% glycerol, 0.05% Toluidine Blue), and then destained for 2 hours with several changes of the same solution without the dye. Pictures were taken after exposing the gel to a white light transilluminator using BioRAD Gel Documentation System.

Results

After apoptosis was induced in the 6-well plates, cells were stained with acridine orange/ ethidium bromide and photographs were taken to see the effects of the different treatments on apoptosis. The acridine orange/ ethidium bromide staining indicated that different treatment agents had different apoptotic potentials which can be seen in Figure 1.

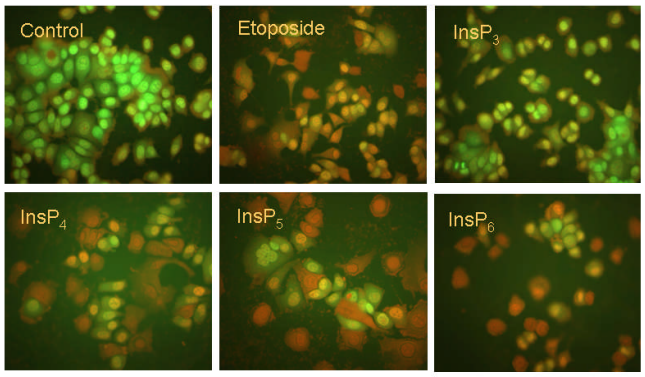

Figure 1: The cells were observed under a UV light using a Nikon fluorescence microscope fitted with a digital camera. The control showed minimal signs of apoptosis, while etoposide showed the most apoptotic (orange) cells. Inositol phosphate treatments at 100µM increased the amounts of apoptosis; however, IP_6 among all inositol phosphates tested showed the most apoptosis.

Quantitative data based on the results in Figure 1 is shown in Figure 2. Etoposide used as positive control induced most apoptosis as has been previously established in our lab (Rakhee et al 2009). In addition, it can also be seen that IP_6 induces more apoptosis than any other inositol phosphates tested.

In order to detect inositol phosphates on PAGE, different IP standards measuring 15nmol each of IP_4, IP_5, IP_6 and IP_7 were run on the 33% polyacrylamide gel. As shown in Figure 3, we can clearly see that IP_6 was detected when the gel was observed under the white light transilluminator after staining with toluidine blue. However, IP_4, IP_5 and IP_7 were not detected. Toluidine blue staining could only detect IP_6 on PAGE under experimental conditions stated in this method. Other experimental conditions and staining procedures may be required to visualize other inositol phosphates on the gel.

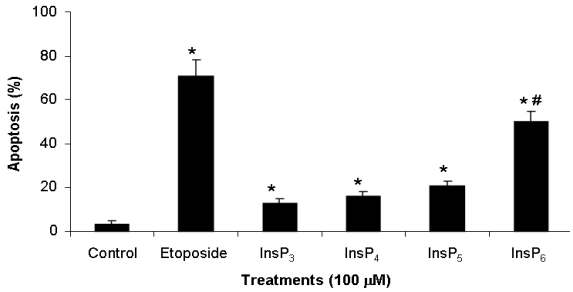

Figure 2: The quantitative analysis of results from Figure 1. Etoposide showed the most apoptotic activity. IP6 was most effective among all inositol phosphates.

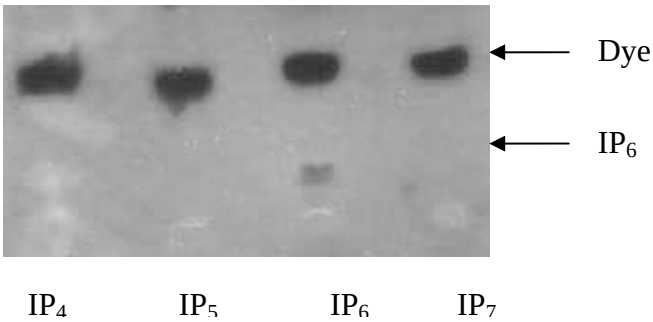

Figure 3: Detection of inositol phosphates (IP_4, IP_5, IP_6, IP_7) standards along with 6x dye were loaded on the 33% gel. We can see the dye front and also the detection of IP_6.

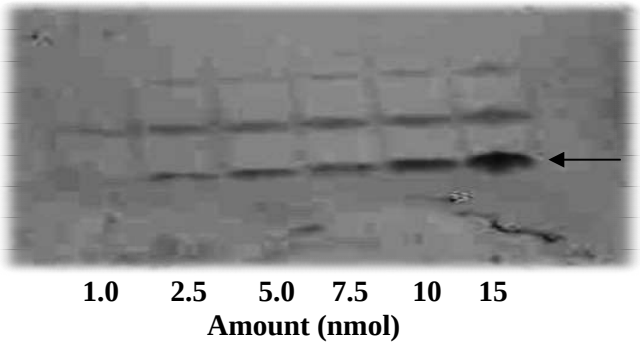

Figure 4: Detection of IP_6 by PAGE. Different amounts of IP_6 ranging from 1.0 nmol to 15.0 nmol were mixed with the 6x dye and loaded onto the gel. Following electrophoresis, gel was stained with toluidine blue. Arrow indicates the position of IP_6. Intensity of IP_6 band increases with amount loaded.

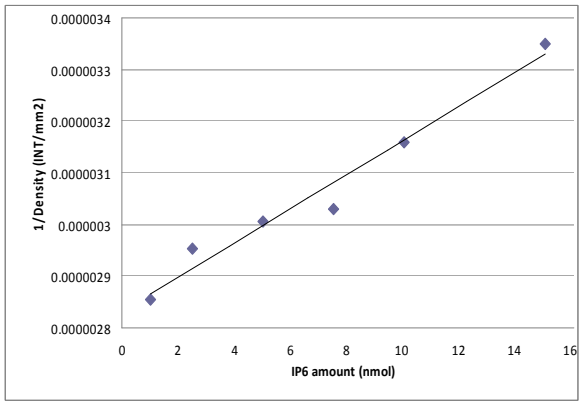

Figure 5: Densitometry Analysis of the Detection of IP_6 by Toluidine Blue Staining. This graph represents the quantitative analysis of the density of the IP_6 bands in Figure 4. The graph shows the linear relations of the detectable amount of IP_6 and density.

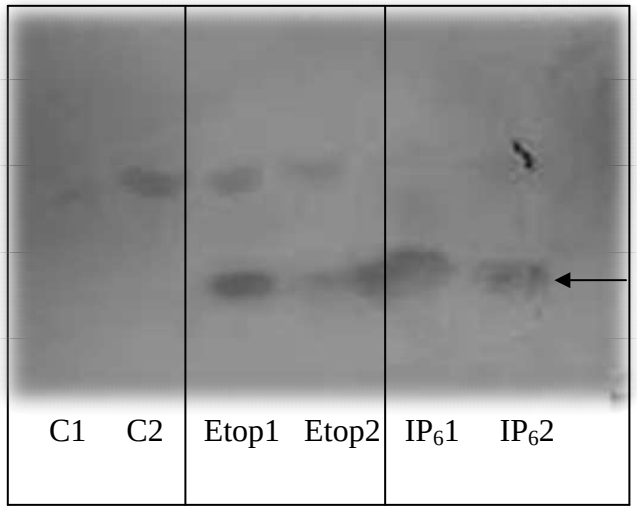

Figure 6: Accumulation of IP_6 during apoptosis induced by etoposide. 100 µl cell extracts were loaded on control and etoposide lanes. The C1 and C2 are control cell extracts (left) showed no IP_6, while etoposide treated cells (Etop1 and 2) showed IP_6 levels which were comparable to the standard IP_6 ($IP_6$1 & $IP_6$2). Arrow shows the position of IP_6.

Since IP_6 is detectable by this procedure, we wondered whether the detection of IP_6 was concentration dependent. Therefore different amounts of IP_6 were prepared, mixed with loading dye and run on a 33 % acrylamide gel. Six different amounts were prepared ranging from 1.0 nmol to 15 nmol**.** Figure 4 shows that IP_6 was separated and the detection was concentration dependent. Densitometry analysis showed that there was a linear relationship between the concentration of IP_6 and the density of the bands observed (Figure 5).

To determine if changes in inositol phosphates levels during apoptosis could be detected, cell extracts from control cells and etoposide-induced apoptotic cells from duplicate samples were prepared and run on the gel along with IP_6 standard. Samples containing extracts or 15 nmol of IP_6 standards were prepared and analyzed as detailed above. Figure 6 shows that no IP_6 was detected in the control cells while apoptotic cells showed the presence of IP_6. We assumed that this band represents IP_6 based on its relative migration on the gel with standard IP_6. Further experiments to hydrolyze the cell extracts with phytase would confirm the identity of this band as IP_6.

Discussion

Inositol phosphates have attracted increased attention for their possible roles as signaling molecules and a wide range of biological functions (Berridge et. al 1989). Similarly, important human diseases such as cancer and diabetes appear to be under regulation by inositol phosphates (Nagata et al, 2005). However, significantly more research is needed to discover the full mechanisms controlled by these signaling molecules. The objective of this study was to examine whether we could detect any changes in IP_6 during apoptosis. According to our results, IP_6 was easily detected compared to other inositol phosphates but the possibility of their detection using PAGE method cannot be ruled out. For example, using the 6x Orange G dye and DAPI staining could possibly enable the detection of IP_7. Alternatively more sensitive detection techniques might help visualize the presence of higher inositol phosphates (IP_7). As far as IP_6 is concerned, the PAGE method used proved to be useful for quantitative analysis of IP_6. Furthermore, under apoptotic conditions, IP_6 levels increased and were detectable by this method.

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