Two-dimensional polyacrylamide gel electrophoresis analysis of indomethacin-treated human colon cancer cells

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AIM: To establish the two-dimensional gel electrophoresis (2-DE) profiles of indomethacin (IN)-treated human colon cancer cell line HCT116, and to provide a new way to study its anti-tumor molecular mechanism through analyzing a variety of protein maps.

METHODS: Two-DE profiles of HCT116 were established in IN-treated and untreated groups. Total proteins were separated by immobilized pH gradient-based 2-DE. The gels were stained by silver, scanned by ImageScanner, and analyzed with Image Master software.

RESULTS: Clear background, well-resolved and reproducible 2-DE patterns of HCT116 cells were acquired in IN-treated and untreated group. The average deviation of spot position was 0.896±0.177 mm in IEF direction and 1.106±0.289 mm in SDS-PAGE direction respectively. In IN-treated group, 1 169±36 spots were detected and 1 061±32 spots were matched, the average matching rate was 90.6% in three gels. In untreated group, 1 256±50 spots were detected and 1 168±46 spots were matched, the average matching rate was 93.0% in three gels. Forty-five differential protein spots were displayed between IN-treated and untreated groups. Of which, 34 protein spots decreased and 9 showed higher expression in IN-treated group, and only two protein spots showed an expression in untreated cells.

CONCLUSION: Two-DE profiles of IN-treated and untreated HCT116 cells were established. Apparent 45 different protein spots were detected in IN-treated and untreated HCT116 cells. The analysis on differential protein spots may serve as a new way to study the molecular mechanism of IN-treated colon cancer.

Key words: Gel electrophoresis; Indomethacin; Colon cancer

INTRODUCTION

Colorectal cancer is one of the most common malignant tumors in the world. It has a high 5-year mortality rate and a low early stage diagnosis rate. It still has a poor prognosis, because 50% of the cases are incurable at the time of diagnosis. It is crucial to find the drugs which have a lower toxicity and a higher effect in treating colorectal cancer. Indomethacin (IN) belongs to nonsteroidal anti-inflammatory drugs (NSAIDs). NSAIDs including IN have many functions such as anti-inflammation, anti-rheumatism, decreasing cerebral thrombosis, and abroad anti-tumor. In addition, using NSAIDs may prevent adenoma occurrence or progression and may lead to a lower incidence of colorectal cancer[6-9]. Several studies have reported a 40-50% decrease in mortality from colorectal cancer with prolonged use of NSAIDs[8-10]. The well-documented pharmacological action of NSAIDs is to inhibit COX[10], but NSAIDs in general have numerous targets other than COX by which they may inhibit tumor cell growth and induce apoptosis, such as modulation of Ras signal transduction, p53 point mutation and activation, of the transcription factor nuclear factor κB[10-15]. But the molecular mechanism by which IN exerts its effects is not well understood. Proteome technology is a useful tool for the identification of new cancer markers and treatment-related changes in cancer. Comparative analysis of protein alterations between pre-treated and treated cells or tissues using high-throughput proteome technology has allowed us to find the special treatment-related proteins and develop new molecular-based therapies. Two-dimensional gel electrophoresis (2-DE) has become the most widely used method for separating and quantifying many types of proteins including those from whole cell lysates, tissue extracts, and subcellular fractions (including membrane proteins). Indeed, 2-DE is regarded as the most powerful separation method for resolving complex mixtures of proteins, and has provided a new way to study the molecular mechanism of IN-treated colon cancer. In this work, 2-DE, followed by ImageMaster analysis was used to study a
variety of protein maps on IN-treated and untreated HCT116 cells. It will provide a new method to look for the correlated proteins of IN-treated colon cancer.

**MATERIALS AND METHODS**

**Materials**

IN, urea, iodoacetamide, acetic acid, DTT and second-dimension SDS-PAGE standard proteins were obtained from Sigma. RPMI1640 and 10% FBS were purchased from Xiangya Medical Collage, Central South University. Acrylamide, agarose, glycerol, bromophenol blue, Tris, CHAPS, SDS, IPG buffer, and the linear immobile dry strips, pH gradients 3-10 (24 cm long) were obtained from Amersham Pharmacia Biotech. Glycine and ammonium persulfate were from Bio-Rad, MTT from Fluk.

**Cell culture**

Human colon cancer cell line HCT116 was obtained from ATCC of USA. The cell line was cultured in RPMI1640 medium supplemented with 10% fetal calf serum at 37 °C, 50 mL/L CO₂ environment.

**Assay of growth-inhibitory effect and IC₅₀ on HCT116 cells**

Growth-inhibitory effect on HCT116 cells was determined by MTT. Cells were planted on 96-well culture plates (2×10⁴ cells/well). After being cultured for 24 h, the cells adhered to the plates. IN was added at 100, 200, 400 and 800 µmol/L for HCT116 cells and cultured for 48 h. IN was dissolved in DMSO such that the final concentration of DMSO was less than 0.2%. One line treated by the appropriate concentration showing 50% cell growth inhibition as compared with control cell growth. The experiments were repeated in triplicate, and the percentage of cell viability was calculated by multiplying the absorbance ratio of the sample vs the control by 100. IN IC₅₀ was determined as IN concentration showing 50% cell growth inhibition as compared with control cell growth. The experiments were repeated in triplicate, and the percentage of cell viability was expressed as mean±SD.

**Time-dependent growth-inhibitory effect of IN (316 µmol/L) on HCT116 cells**

HCT116 cells were seeded in 96-well plates (2×10⁴ cells/well) in RPMI1640 medium with 10% FBS. After 24 h, media were replaced with fresh standard media or media containing 316 µmol/L IN. According to MTT method, at the appropriate time points, the absorbance of each well was determined by a spectrophotometer at 490 nm wavelength. The percentage of all viability was calculated by multiplying the absorbance ratio of the sample vs the control by 100.

**Sample preparation**

HCT116 cells were seeded in 75-cm² tissue culture flasks, grown for 1-2 d prior to use. When 50% confluent growth was reached, the media were changed to fresh standard media or media containing 316 µmol/L IN. The cells were harvested 48 h after treatment, rinsed with PBS (0.8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L NaH₂PO₄, 0.24 g/L KH₂PO₄), and trypsinized with a solution of 2.5 g/L trypsin and 0.2 g/L EDTA. After 1 min, media containing FBS were added to terminate the action of trypsin. The resulting suspension was centrifuged at 1 000 r/min for 7 min at 4 °C. After the supernatant was discarded, the cells were resuspended in ice-cold 1× PBS and centrifuged at 1 500 r/min for 10 min at 4 °C and the supernatant was removed. This wash-step was repeated thrice and stored at -80 °C until further use.

Protein extraction from cells untreated and treated with IN was performed with lysis buffer. The harvested IN-treated and untreated HCT116 cells were left in lysis buffer (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 40 mmol/L Tris and 1 mmol/L PMSF) for 30 min in ice. The resulting cell lysates were then vortexed. The sample was incubated at room temperature for 10 min. After centrifugation at 15 000 g at 4 °C, for removal of particulate materials, the protein solution was collected and stored at -80 °C until use.

Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad) with BSA (Sigma) as the standard.

**IPG-2D PAGE**

IPG-IEF was run on an IPGphor isoelectric focusing (IEF) system (Amersham Pharmacia Biotech). Twenty-four centimeter pH 3-10 immobilized pH gradient strips were rehydrated for 14 h with 450 μL of 2-D solubilizing solution (8 mol/L urea, 2% CHAPS, 0.5% IPG buffer, pH 3-10, 3% DTT and a trace of bromophenol blue) containing 260 µg of total proteins for analytical runs, and mixed with a rehydrated solution to a total volume of 450 μL. The mixtures were pipetted into IPG strip holder channels. IPG dry strips were lowered into the mixtures with the gel side down, and overlayered with the dry-strip fluid covered. The holders were placed onto the electrode plates of the IPGphor platform. After rehydration for 14 h, IEF was carried out with a low initial voltage (500-1 000 V) during the first 2 h and then the voltage gradient up to 8 000 V with a limiting current of 15 μA/strip. The total product time×voltage applied was 69 920 V·h for analytical runs. The temperature was maintained at 20 °C. Following IEF separation, the gel strips were equilibrated for 2×15 min in an equilibration buffer containing 50 mmol/L Tris-HCl, pH 8.8, 6 mol/L urea, 30% glycerol, 2% SDS and a trace of bromophenol blue. One percent DTT was added to the first equilibration buffer, and 2.5% iodoacetamide was added in the second equilibration buffer. The equilibrated gel strips were then applied onto 0.75-mm thick 12.5% SDS linear polyacrylamide gradient vertical slab gels, and sealed with 0.5% agarose. SDS-PAGE was run in a Bio-Rad Protein II electrophoresis apparatus for 30 min at constant current of 10 mA/gel, and then switched to 25 mA/gel until the bromophenol blue frontier reached the bottom of the gels. During the whole run the temperature was set at 15 °C. To determine the isoelectric point (pI) and molecular weight (M) of separated proteins, 2-D standards were added to the protein samples as internal markers. After 2-DE, the protein spots were visualized by silver-based staining technique with the protein silver stain kit (Amersham Biosciences).

**Image analysis**

The stained 2-DE gels were scanned with LabScan software.
on ImageScanner (Amersham Pharmacia Biotech). The spot-intensity calibration, spot detection, background abstraction, matching, 1-D calibration, and the establishment of average-gel were performed with ImageMaster 2D Elite 4.01 analysis software (Amersham Pharmacia Biotech). Intensity of each spot was quantified by calculation of spot volume after normalization of the image using the total spot volume normalization method multiplied by the total area of all the spots. The reproducibility of spot position was calculated according to Corbett’s method[21].

Statistical analysis
Data were expressed as mean±SD with the exception of 2-DE data, which were expressed as mean only. Data were analyzed using parametric Student’s / test. Statistical analysis was carried out with SPSS for Windows 11.0. P<0.05 was considered statistically significant.

RESULTS
Effect of IN on HCT116 cells
MTT assay demonstrated that HCT116 cells were sensitive to IN. Dose-dependent growth-inhibitory effect of IN on HCT116 cells was found at the concentrations of 0, 100, 200, 400, 800 μmol/L (Figure 1A). From the growth-inhibition curve figure, we got IC₅₀ = 316±1.2 μmol/L. This analysis revealed an increase in toxicity with increasing IN concentration. Cells were exposed to 316 μmol/L of IN for varying times (12, 24, 36, 72 h) at which the IN-containing medium was replaced and/or the proliferation assay was performed. There was time-dependent growth-inhibitory effect of IN that showed a significant growth inhibition (Figure 1B).

Result of 2-DE and ImageMaster analysis
Resolving power
Total cell lysate was isolated from IN-treated and untreated HCT116 cells and analyzed by high-resolution 2-DE. IEF was first conducted on a 24 cm, pH 3-10 nonlinear immobilized pH gradient (IPG) strip. For colon cancer HCT116 cells, 1213±58 spots were detected.

Reproducibility and expression of 2-DE profiles on IN-treated and untreated HCT116 cells
With the same condition and parameters, the experiment was repeated thrice from cell culture to 2-DE respectively. For IN-treated and untreated HCT 116 cells, the clear background, well-resolved and reproducible 2-DE pattern were attained (Figure 2). Through Image Master 2D Elite 4.01, we found that the separation was better in the lower molecular weight range and towards the acidic pH. Horizontal streaks were apparent for proteins in the higher molecular weight range. Three silver-stained 2-DE profiles of each sample were very similar. The image analysis showed that these 2-DE maps were reproducible. For IN-treated HCT116 cells, the average spots were 1 169±36 in three gels, 1 061±32 spots were matched with an average matching rate of 90.6%. For untreated HCT116 cells, the average spots were 1 256±50 in three gels, and 1 168±46 spots were matched with an average matching rate of 93.0%. A total of 100 well-resolved and matched spots among three untreated cells were chosen randomly to calculate the deviation of the spot positions. The spot positional deviation was 0.896±0.177 mm in IEF direction and 1.106±0.289 mm in SDS-PAGE direction. A total of 1 058±47 spots were matched in IN-treated-2-DE and untreated-2-DE maps. Compared with untreated maps, average spots decreased 6.9% in IN-treated group.

Figure 1 Dose-dependent grow-inhibitory effect (A) and time-dependent growth-inhibitory effect (B) of IN on HCT116 cells.

Figure 2 Two-dimensional electrophoretic maps of human colon cancer HCT116 cells untreated (A) and treated with IN (B). + stands for differential protein spots. 1-34 means decreasing spots and 35-43 increasing spots in IN-treated cells. Forty-four and 45 spots were expressed in untreated cells.
In this study, the 2-DE protein patterns of IN-treated and untreated pairs were quantified and matched mutually. Furthermore, the differentially detected protein spots between IN-treated and untreated group in three experiments must accord with a significant difference in relative volume ($P<0.05$, Table 1). Forty-five differential protein spots were displayed between IN-treated and untreated groups. Of which, 34 protein spots decreased and 9 showed higher expression in IN-treated group, and only 2 protein spots showed an expression in untreated cells.

### DISCUSSION

It is a hot point of using IN in anti-neoplastic therapy, but the molecular mechanism by which IN exerts its effects is not well understood. Subbegowda and Frommel [22] found in flow cytometric analysis, an increase of cells and subdiploid nuclei in S and G2/M phases in cultures treated with high dose of aspirin. The results suggested that aspirin could induce cell cycle arrest and cause necrosis at high concentrations in vitro, but not apoptosis. In our previous study, IN altered the cell cycle phase distribution of HCT116 cells, and
increased the proportion of cells in the G0/G1 phase and reduced the proportion in the S phase of the cell cycle. In addition, we proved that IN could induce apoptosis and found that the anti-tumor activity of IN through P35-P21/WAF1/CIP1 depended on pathways to inhibit cell proliferation and induce cell apoptosis\cite{25,26}. Compared with untreated maps, the average spots decreased 6.9% in IN-treated group in our study. According to MTT experiments, we confirmed that IN could inhibit the proliferation of HCT116 cells.

The expression of proteins is different in different tissues, cells, and stage of upgrowth, physiology or pathology. The basic challenges are to find the proteins and to predict their functions. This would contribute to a new understanding of human biology and to the design of new molecular structures as potential novel diagnostic or drug discovery targets\cite{27}. In order to further explore the molecular mechanism of IN in treating colon cancer, we used high-throughput proteomics technique to address the molecular basis of this effect through analyzing protein expression profiles of HCT116 cells in treated and untreated groups. In our experiment, IPG-IEF was run on an IEF system. For the sample swelling in with hydration fluid and rehydrated and isoelectrically focused in the same chamber, we avoided the use of sample cups, eliminated precipitations at the sample application site, thus improving resolution over the entire pH range of the gels. It also allowed precise control of protein amounts and sample volumes loaded onto the IPG gels, and also enlarged the reproducibility of 2-DE\cite{20,21}. In addition, we used the newest Pharmacia software, ImageMaster, to analyze our protein maps. Compared with Bio-Rad PDQuest, ImageMaster 2-D gel analysis software (4.01 version) demonstrated a higher sensitivity. From the ImageMaster, we found that most of protein spots distribute pI: 3-8 and M: 20.1-66 ku. We also found that the separation was better in the lower molecular weight range and towards the acidic pH. Horizontal streaks were apparent for protein in the higher molecular weight range. This seems to be a common feature of 2-DE, seen in most gels\cite{22,23}.

Clear background, well resolved and reproducible 2-DE patterns of IN-treated and untreated HCT116 cells were acquired. Three silver-stained 2-DE profiles of each sample were very similar. The image analysis showed that the 2-DE maps were reproducible. A total of 1 256±36 protein spots were resolved in untreated group and the match rate was 100%. A total of 1 169±36 protein spots were resolved in IN-treated group and the match rate was 90.6%. In order to further identify our experiment that was reproducible, a total of 100 well-resolved and matched spots in the IN-treated group were chosen randomly to calculate the deviation of the spot positions. The spot positional deviation was 0.896±0.177 mm in IEF direction and 1.106±0.289 mm in SDS-PAGE direction. Analyzing the 2-DE profiles of IN-treated and untreated groups, 45 differential protein spots displayed quantitative changes in expression after IN treated. Of which, 34 decreased in abundance, 9 showed a higher expression, and only 2 were expressed in untreated cells. The results indicated that there were many pathways of IN in colon cancer. Most of pI values distributed pH 3-8 in our gels. Molecular weight distributed 21-61 ku. Comparison of the differential protein spots between IN-treated and untreated groups could contribute to study the molecular mechanisms of the anti-tumor effect of IN. Some differential protein spots between IN-treated and untreated cells should be further identified with amino acid sequence analysis and mass spectrometry analysis.

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