EXPERIMENTAL STUDY

Adenosine A1 receptor modifies P53 expression and apoptosis in breast cancer cell line MCF-7

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ABSTRACT

BACKGROUND: Breast cancer cells over-express the adenosine receptor A1 and in most of these cells, P53 gene is a wild type. Because of this finding and relationship between A1 receptor and cell apoptosis and proliferation, this study aimed to determine the effect of agonist and antagonist of A1 receptor on cell apoptosis and proliferation and recognize the relationship between this receptor and P53 expression.

METHODS: We used a Real-Time PCR test for measuring expression of p53 gene also flow cytometry assay for apoptotic and survival cell rate after treatment of MCF-7 cells with A1 receptor agonist CPA (N6-Cyclopropyladenosine) and A1 receptor antagonist DPCPX (1,3-dipropyl-8-cyclopentylxanthine) in 24,48 and 72 hours.

RESULTS: Our flow cytometry findings indicate that DPCPX significantly induces apoptosis in MCF-7. Also the expression of P53 becomes upregulated with time of DPCPX treatment. CPA treatment increased the survival cell rate and down-regulated this apoptosis-relevant gene P53 (p > 0.05).

CONCLUSION: DPCPX can induce P53 expression which consequently promotes the cell apoptosis in MCF-7. Therefore, DPCPX could be used as an anti-cancer agent (Tab. 1, Fig. 3, Ref. 5). Text in PDF www.elis.sk.

KEY WORDS: receptor, adenosine A1, apoptosis, gene, p53, MCF-7 cells.

Introduction

Breast cancer is the most frequent malignancy occurring in women in developed countries, where about one in eight women will have a lifetime risk of this cancer and one-third of patients will die of this disease (1, 2).

MCF-7 is one of the breast cancer cell lines that are seen in 70% of patients with breast cancer. In this cell line, P53 is a wild type (3).

Cancer cells use different mechanisms to escape from proliferation and growth control checkpoint while these mechanisms mainly accompany the genomic change in these cells. These changes can lead to abnormal cell proliferation, cell movement, and invasion or even increased drug resistance and other properties of malignant cells.

Changes in the cell signaling pathway can lead to these events and one of these changes is an expression of special receptors (1, 2).

A1 receptor is one of the Purinergic P1 receptors (A1, A2a, A2b, and A3) that generally control immune and inflammatory reactions, apoptotic pathway, and cell division and differentiation. A1 receptor expression and concentration are different in different tissues and cells (4, 5, 6, 7).

Programmed cell death or apoptosis is used for various biological events such as senescence, clearance of excess and harmful cells. This type of death is essential for normal development and homeostasis. Two main apoptotic pathways consist of extrinsic or death receptor-mediated apoptosis and intrinsic or mitochondria-mediated apoptosis (8). Many drugs used for cancer treatment induce apoptotic death through the p53-dependent pathway (9).

P53 is one of the important factors for controlling mitosis and cell proliferation; also well known as a tumor suppressor gene in human genome. In cancers, P53 is a reliable biomarker to determine the prognoses of patients and follow-up treatment results. This molecule has several biological functions including DNA repair, cell cycle control, differentiation, and apoptosis. P53 is mutated in 50% of human cancers but this rate in breast cancer is only 20–30%, and in most cases this gene is a wild type. In addition, P53 is a key initiator in external and internal apoptotic pathways. Inhibition of P53 gene expression can also cause uncontrolled cell division (8, 10).

A1 receptor is expressed in all types of breast cancer cells but so far the signaling pathway of A1 receptor in these cells is not clear (11). Some researchers showed that A1 receptor expression increased the cell viability and mitosis rate and decreased the apoptosis in these cells. This expression is critical also for estrogen receptor function and signaling (12, 11).

Studies show that the activation of A3 receptor with IB-MECA as an agonist for this receptor in prostate cancer leads to P53 overexpression, cell cycle arrest, and caspase-3 activation (13). In the kidney, brain and heart, the activation of A1 receptor reduced cell damage and apoptotic rate following oxidative stress while the
antagonist of this receptor increased cell and tissue damage under these conditions (14). This receptor can inhibit caspase-3 activation as well as apoptosis in hypoglycemic brain (15).

In this study we investigated the effects of A1 receptor agonist (CPA, N6-Cyclopentyladenosine) and antagonist (DPCPX, 8-Cyclopentyl-1, 3-dipropylxanthine) on expression of P53 and apoptotic rate.

**Materials and methods**

**Cell lines, drug, treatment and culture condition**

Mcf-7 cell line obtained from the Pasteur Institute of Animals Cell Culture. Cells were maintained in Dulbecco’s modified Eagle medium (DMEM/F12) with 10 % FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and kept at 37°C in a humidified atmosphere with 5 % CO2. For experiments after trypsinization (Trypsin-EDTA), cells were seeded in culture flasks. CPA and DPCPX was purchased from Sigma (C8031, C101) and dissolved in DMSO to the appropriate concentrations according to IC50 assay. After the cells had been >80% confluent and grown exponentially in 75 mm diameter culture flask, 10⁶ MCF-7 cells were counted and plated in 75-mm diameter culture flask and kept in culture medium for 24 hours which were then incubated with certain concentrations of CPA and DPCPX, based on IC50 index for 24 h and at certain times (24, 48 and 72 h) the tests were performed.

**IC50 assay**

The IC50 values for CPA and DPCPX in MCF-7 groups were acquired after 24 hours of treatment. Briefly, 10⁴ cells (MCF-7) were counted and placed into each well of a 12-well plate and were treated with various drug concentrations (0, 10, 50, 100, 200, 400 μM doses for CPA and 10 nM, 50 nM, 100 nM, 1 μM, 10 μM, 100 μM doses for DPCPX) for 24 hours, and the MTT survival assay was then carried out for evaluating the cell viability with different drug concentrations in each groups. A graph of cell viability versus drug concentration was used to calculate IC50 values for MCF-7 cell line.

**Real-Time PCR**

Total RNA from cells was extracted at the indicated times, using the RNeasy mini kit and following the instructions from the manufacturer (Qiagen, Hilden, Germany). After treatment with DNase I, Total RNA (100 ng) was reverse-transcribed to cDNA by using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer’s instructions. The Maxima SYBR Green Rox qPCR master mix kit (Fermentas) was used for real-time RT-PCR. Primer sequences are shown in Table 1. Real-time PCR reactions were performed with StepOnePlus (Applied Biosystem). The program of Real-Time PCR consisted of 10 min at 95 °C followed by 40 cycles of denaturation step at 95 °C for 15 sec following by annealing and extension for 1 min at 60 °C. Data were analyzed by Comparative Ct (ΔΔct) method. The relative expression level of p53 was calculated by determining the ratio between the amount of p53 and that of endogenous control. Melting curve analysis (60 °C → 95 °C increment of 0.3 °C) was used to determine the melting temperature of specific amplification products and primer dimers. These experiments were carried out in triplicate and were independently repeated at least 3 times.

**Flow-Cytometric analysis**

The percentage of apoptotic cells was measured by flow cytometry following AnnexinV (FL1-H) and PI (FL2-H) labeling.

**Tab. 1. Primers used in real-time PCR.**

<table>
<thead>
<tr>
<th></th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GAPDH forward</td>
<td>CACCACCATGGAGAAGGCTGG</td>
</tr>
<tr>
<td>2</td>
<td>GAPDH reverse</td>
<td>CCAAGTGGTCATGGATGACC</td>
</tr>
<tr>
<td>3</td>
<td>P53 forward</td>
<td>CGAGTCCCGGGTAATTCTT</td>
</tr>
<tr>
<td>4</td>
<td>P53 reverse</td>
<td>TGCAAGAGGGTGCAAGACC</td>
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**Fig. 1a, b. IC50 assay for half maximal inhibitory concentration analysis of CPA (1a) and DPCPX (1b) in MCF7 cancer cell lines after 24 h of treatment. Cells were incubated with/or without the drug in different concentrations, and the relative amount of viable cells were estimated by measuring the absorbance of the cell suspension after incubation with MTT assay.** Graph of viability versus drug concentration was used to calculate IC50 values.
A minimum of $4 \times 10^5$ cells/ml were analyzed for each sample. Cells were treated with CPA and DPCPX ($180 \mu M$ and $87 \text{nM}$) for 24 and then in 24, 48, and 72 hours washed in PBS and resuspended in binding buffer ($1\times; 5\mu l$). AnnexinV-FITC was added to $195\mu l$ cell suspensions and then the analysis was carried out according to the manufacturer's protocol (BMS500F1/100CE AnnexinV-FITC, eBioscience, USA). Finally, the apoptotic cells were counted by FACScan flow cytometry (Becton Dickinson, Heidelberg, Germany). These experiments were carried out in triplicate and were independently repeated at least 3 times.

Statistical analysis
All the quantitative data were presented as the mean ± standard deviation. One-way analysis of variance (ANOVA) with LSD post-hoc test was performed to determine statistical significance among different groups by using SPSS software package 16.0. Significance was accepted at a level of $p < 0.05$.

Results

Results of IC50 assay
After the treatment of MCF-7 cells with MTT solution, the dark blue formazan crystals were seen in viable cells, which indicated their metabolic activity. The reduction in the number of cells was dependent on the cell types as shown by the half-maximal inhibitory concentration (IC50) index. Then the IC50 values for the CPA and DPCPX were established (Fig. 1a). The results show the essential concentration of CPA while DPCPX to achieve the IC50 in MCF-7 cells at 24 hours was $180 \mu M$ and $87 \text{nM}$ (Fig. 1b).

Results of Flow cytometry
The flow cytometry assay was used to determine the apoptotic potential of the CPA and DPCPX; these results showed that the $87 \text{nM}$ concentration of DPCPX based on IC50 index at different times (24, 48 and 72 h) could significantly induce apoptosis in MCF-7 cells while it increased with elapsing time ($p < 0.05$) (Fig. 2). DPCPX treatment arrested MCF-7 cell proliferation and induced apoptosis ($\geq 65\%$ of inhibition) in 72 hours, whereas the effect of CPA on cells’ programmed death at all different times was negative ($p > 0.05$) (Fig. 2). MCF-7 apoptotic cells showed a sharp increase at all times in comparison with control group cells ($p < 0.05$). DMSO was used in the control sample (drug vehicle) and a small amount of cell apoptosis in cell line at different times was observed ($p < 0.05$) (Fig. 2).

Results of Real-Time PCR
To examine CPA and DPCPX effects (based on IC50 index) at different times on the mRNA expression of P53 gene on MCF-7 cells we used Real-Time quantitative PCR. The P53 gene expression was dramatically up-regulated by DPCPX treatment with elapsing time in MCF-7 cells, in particular at 72 hours of treatment its expression was significantly raised ($p < 0.05$). CPA significantly down-regulated the genes’ expression at different times ($p > 0.05$), especially at 72 hours (Fig. 3).

Discussion
Recently, several groups have reported the use of cell receptors such as adenosine receptors family for successful controlling or
overcoming resistance to cancer drugs (7). In cancer therapy, the ideal therapeutic approach would be to induce apoptosis specifically to primary tumors, as well as those at the site of metastatic cells while simultaneously monitoring the prognosis through non-invasive approaches (16).

Adenosine A1 receptor is a member of the G protein-coupled receptor (GPCR) super family. Based on different roles of adenosine A1 receptor in some diseases, it has also been suggested that this receptor may act as a potent regulator of the growth of normal and tumor cells by exerting pro- and anti-apoptotic or pro-survival effects (17, 18, 19). It was suggested earlier that apoptotic induction in cancer cell lines by adenosine receptor A1 antagonist requires the activation of P53 gene expression (2, 10). In this study, responses of MCF-7 cells against agonist and antagonist of A1 receptor were investigated.

Our results in flow cytometry assay demonstrated that the use of adenosine A1 receptor antagonist (DPCPX) induced apoptosis in MCF-7 cells and adenosine A1 receptor agonist (CPA) decreased apoptosis rate. MTT assays revealed that DPCPX exerts significant cytotoxicity to MCF-7 cells in time-dependent fashion in the concentration range of 87nM. Furthermore, quantitative Real-Time PCR analysis displayed that mRNA level of P53 involved in the apoptosis was altered by this treatment. Overall, our data suggest that A1 receptor antagonist may induce apoptosis in MCF-7 cells via p53 over-expression. This in vitro study showing the induction of apoptosis by A1 receptor antagonist warrants further investigation to determine whether in vivo treatment consequences may exist for this receptor application.

In conclusion, we have shown that A1 receptor antagonist (DPCPX) produce significant apoptosis in MCF-7 cells in the concentration range of 180 nM. Furthermore, quantitative Real-Time PCR analysis displayed that mRNA level of P53 involved in the apoptosis was altered by this treatment. Overall, our data suggest that A1 receptor antagonist may induce apoptosis in MCF-7 cells via p53 over-expression. This in vitro study showing the induction of apoptosis by A1 receptor antagonist warrants further investigation to determine whether in vivo treatment consequences may exist for this receptor application.

This role is mediated by microglia cells (22). But so far, no evidence has been reported that A1 adenosine receptors induce P53 expression and apoptosis, conversely, it has been shown that these receptors decrease apoptotic cell death in some cells (23, 24). Our findings oppose K. Sai et al results from astrosytoma RCR-1 cell line which show that A1 adenosine receptor agonist (CHA) induces apoptosis by activating caspase-9 and the ensuing caspase-3 via being linked to A1 adenosine receptor in RCR-1 cells (25).

In cancer, targeted therapy is one of the key challenges that make use of agents specific for the deregulated proteins of cancer cells in each site of body such as specific receptors and generally deactivate the enzymatic domains on mutated, over-expressed, or otherwise critical proteins within the cancer cell (16). Therefore, special cell receptors have the potential to play a key role in achieving such goals. It is anticipated that A1 receptor antagonist might significantly reduce the usage of anti-cancer drugs with better specificity, enhanced efficacy and lower toxicities and side effects because it is targeted on special cells only.

In conclusion, we have shown that A1 receptor antagonist (DPCPX) produce significant apoptosis in MCF-7 cells in the concentration range of 180 nM. Furthermore, quantitative Real-Time PCR analysis displayed that mRNA level of P53 involved in the apoptosis was altered by this treatment. Overall, our data suggest that A1 receptor antagonist may induce apoptosis in MCF-7 cells via p53 over-expression. This in vitro study showing the induction of apoptosis by A1 receptor antagonist warrants further investigation to determine whether in vivo treatment consequences may exist for this receptor application.

References


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