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INVESTIGATION OF THE EFFECTS OF PROTOCATECHUIC ACID ON C6 CELL PROLIFERATION USING ANTI-PCNA PRIMARY ANTIBODIES

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Abstract

Cancer is the abnormal growth of cells. Cancer cells multiply rapidly despite space constraints, nutrients shared by other cells, or signals from the body to stop reproduction. Cancer cells are often shaped differently from healthy cells, do not work properly, and can spread to many parts of the body. Tumors are abnormal tissue growth, clumps of cells that can grow and divide uncontrollably.

In this article, the anti-proliferative effects of protocatechuic acid (PCA) concentrations determined as a result of cytotoxicity test on C6 rat glioma cells in two different ways were investigated. For this, PCNA immunofluorescence method was used. The effects of protocatechuic acid on C6 cell proliferation were examined using anti-PCNA primary antibody on at least 30 photos from each group. The values of the fluorescent intensity ratios of PCNA-labeled cells between the experimental groups were statistically compared with the control group. Compared to the control group, it was observed that cell proliferation decreased significantly at increasing concentrations both in the groups in which PCA was applied to C6 cells alone and in the groups in the inflammation culture model created with THP-1 human monocyte cells.

Keywords: Cancer, C6, PCA, PCNA.

1. Introduction

PCNA (proliferating cell nuclear antigen) is a protein with a molecular weight of 36 kDa (kilodalton) that functions as a cofactor of DNA polymerase delta. Information about PCNA was first given by Miyachi in 1978. The presence of antibodies that react with proliferating cell antigens in the serum of patients with systemic lupus erythematosus has been reported. After this, Bravo and Celis observed in their research in 1980 that a protein they called "cyclin" showed different effects in the cell cycle. Mathews et al. revealed that PCNA and cyclin are actually the same protein [11, 3, 5].

Visualization of the nuclear distribution of PCNA (via antibody labeling) can be used to distinguish the early, middle and late S phase of the cell cycle. In proliferating cells, PCNA appears in the G1 phase of the cell cycle, starting to rise in the middle of this phase and remains elevated throughout the S phase. In G2 and M phases, however, it decreases to the same level in G1 phase [14, 6].

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PCNA is a homotrimer and achieves its processing capability by surrounding DNA, where it acts as a scaffold for recruiting proteins involved in DNA replication, DNA repair, chromatin remodeling, and epigenetics [12].

Detection of PCNA as a result of immunohistochemical studies indicates both active DNA replication and the presence of DNA damage resulting in carcinogenesis. PCNA has been successfully demonstrated in the nuclei of human glioma cells both in vitro and in situ by an indirect immunoperoxidase method. Spotted nucleoplasmic staining for PCNA with variable nucleolar staining was observed in approximately 37% of cultured human glioma cells in exponential growth, whereas only 14% of subcutaneous tumor cells from vaccinated nude rats stained definitively positive for nuclear PCNA [13]. It has also been shown in many studies that it is used as a proliferation marker in liver, gastrointestinal system, breast, skin, urinary system and lung malignancies and provides tumor infiltration [8, 1]. Although PCNA expression is expected to increase in proliferating cells, it can sometimes be observed in non-proliferating cells when there is active DNA repair in many tumor cells [4].

2. Material and Method

In order to determine the effects of protocatechuic acid on C6 cell proliferation, immunofluorescence labeling was performed using anti-PCNA primary antibody (PC10, Mouse mAb Cell Signalling Technology, 2586) and Alexaflor488 (Cell Signaling Technology 4412) secondary antibody.

When C6 rat glioma cells were grown in culture medium and reached sufficient cell density, the cells were removed from the surface to which they adhered and counted. After counting, 1 x 10^6 cells per well were seeded in a 6-well culture plate and incubated until the cells formed a monolayer (approximately 24 hours). After incubation, the medium of the cells was withdrawn from the wells and washed once with 1x PBS. After the washing process, the control group, positive control (Karmustine 300µM) and appropriate concentrations of PCA (3.8 and 5 mM) were applied to the wells in the appropriate medium [2] and the cells were left for 24 hours incubation again.

- ✓ After incubation, the medium of the cells was removed and then washed 3 times with 1XPBS.
- ✓ After cells were washed, they were fixed in each well with 4% paraformaldehyde (PFA) in 1XPBS for 20 min.
- ✓ After fixation, it is washed again 3 times with 1XPBS.
- ✓ Permeabilize with Triton X100 for 15 min in 1 X PBS containing 1% BSA.
- ✓ Cells were washed 3 times with 1XPBS and the appropriate primary antibody solution diluted 1:2000 with 1% BSA in PBS buffer was added and incubated overnight at +4 °C.
- ✓ Secondary antibody: Anti-rabbit IgG (H+L) F (ab)2 fragment washed 3 times with 1XPBS and diluted with 1% BSA in PBS buffer was incubated with Alexa flour 488 conjugate (Cell Signaling Technology) for 2 h. 4412). This step was performed in the dark.
- ✓ Finally, it was washed 3 times with 1XPBS followed by distilled water, and the plate was placed in a Cytation 3 Cell Imaging Multi-Mode Reader and fluorescence images of the cells were captured.

Randomly selected fields from at least 30 photographs from each group were examined, the results were analyzed in ImageJ Imaging Software, and fluorescence intensity levels were determined. Results for fluorescence intensity are expressed in representative units.

Statistical analysis

Results were calculated as mean±standard deviation. Experiments were performed in 3 replicates (n:8). The % values of cell viability compared to the control group were calculated in Microsoft Office Excel. GraphPad Prism 6.0 software and SPSS Statistics 20 software were used for statistical evaluations and graphs. The obtained data were analyzed by applying one-way ANOVA and post-hoc Tukey's test. Importance values; $p>0.05$ no difference, $p<0.05$* difference, $p<0.001$*** significant difference and $p<0.0001$**** very significant difference.

3. Discussion

3.1. Cell lines. Rat glioma cells (C6) were grown in culture medium in Dulbecco's Modified Eagles Medium (DMEM) growth medium containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (PS), and 0.1% pyromycin (Fig. 1A). Human monocyte cells THP-1 in culture medium of Roswell Park Memorial Institute 1640 (RPMI-1640) growth medium containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (PS), 0.1% pyromycin, and 0.0004% β-mercaptoethanol reproduced. In accordance with the protocol, first M0 and then M1 were differentiated into macrophages (Fig. 1B).

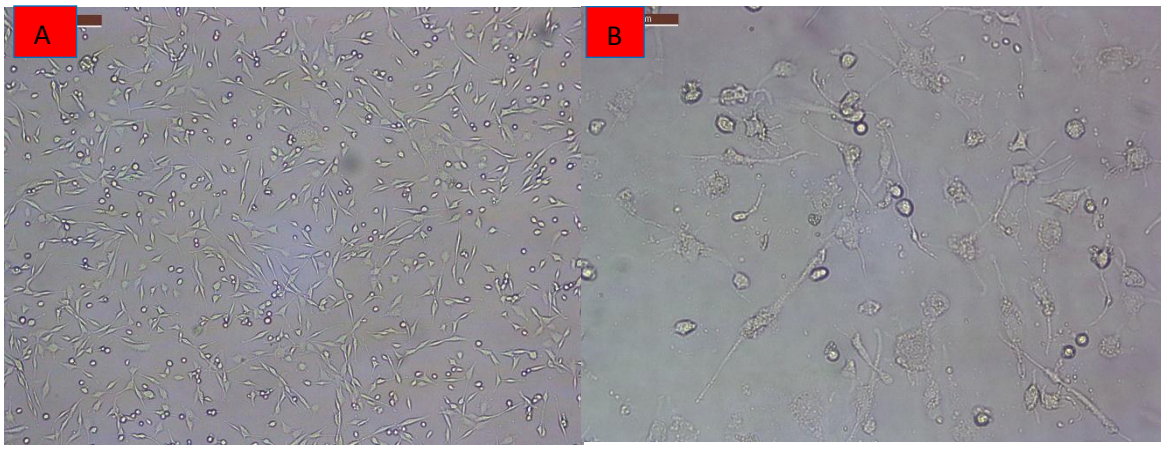

Fig. 1. Images of the cells used under the microscope;(A) C6 rat glioma cells, (B) M1 macrophages from THP-1.

3.2. Evaluation of Immunofluorescence *Assay with Proliferating Cell Nuclear Antigen (PCNA).* The effects of protocatechuic acid on C6 cell proliferation were studied using anti-PCNA primary antibody (PC10, Mouse mAb Cell Signaling Technology, 2586) on at least 30 photos from each group. As seen in Fig. 2, when the fluorescence intensity ratios of PCNA-labeled cells were compared statistically with the control group, it was observed that cell proliferation decreased significantly in both groups (C6 cells alone and at increasing concentrations of the groups treated with M1 cytokine medium) between the experimental groups.

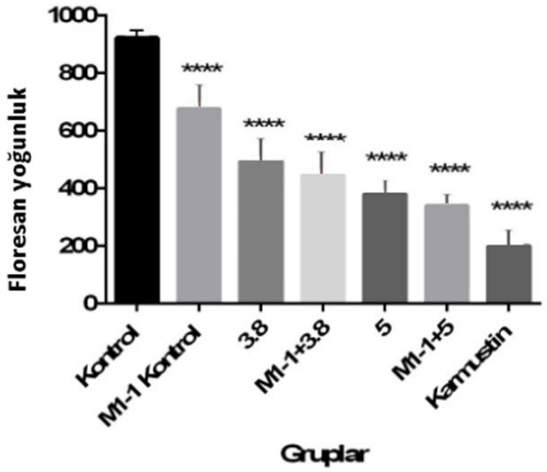

Fig. 2. *Graph of statistical evaluation of PCNA expression on C6 cell proliferation of PCA:(p<0.05*, p<0.01**, p<0.001***, p<0.0001****, Mean ± Standard Deviation, n=30) .*

4. Conclusion

In our study, the effects of protocatechuic acid on C6 cell proliferation were investigated using PCNA, a proliferation marker, a proliferating cell nuclear antigen. 3.8 and 5 mM concentrations of protocatechuic acid were used. As a result, it was observed that cell proliferation was significantly reduced in both the groups in which PCA was applied to C6 cells in normal medium compared to the control group, and the increased PCA concentrations of the groups in which M1 macrophage cytokine medium was applied. In a study on PCNA expression of protocatechuic acid in in vitro cell culture, it was determined that 1, 1.5 and 2 mM PCA concentrations increased the expression of proliferating cell nuclear antigen (PCNA) in RSC96 rat neuronal Schwann cells [9].

In the study of Kou et al. examining the inhibitory effect of caffeic acid phenethyl ester on the growth of C6 glioma cells in vitro and in vivo, PCNA immunohistochemistry as a marker of cell proliferation was examined. As a result, it was shown that the number of PCNA positive cells in C6 cells was significantly reduced compared to the control group [10].

Hirose et al., in their in vivo study, determined that PCA significantly reduced PCNA expression in liver tissue in Fischer-344 rats [7].

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