



Original Contribution

CHARACTERIZATION OF THE PROLIFERATIVE ACTIVITY OF THE SERUM-FREE CELL LINE MCCOY-PLOVDIV

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ABSTRACT

The aim of the present study was to examine the proliferative potential of the new serum-free cell line McCoy-Plovdiv. We used the biotin-streptavidin-peroxidase system to determine immunocytochemically the expressions, over time, of PCNA, Ki67 and EGFR on this new cell line. PCNA was detected under the nuclear membrane at 1st hour of culturing. It peaked at 72nd hour and declined after Day 8. The nucleoli showed Ki67 at 24th hour but the expression declined after Day 6. All cells in subconfluent cultures expressed EGFR. Confluent cultures, however, showed reduced EGFR expression in both cell number and intensity. Our results showed that PCNA was an early and informative marker of the proliferative activity of the new serum-free cell line McCoy-Plovdiv. Ki67 was not a sufficiently informative marker due to its late expression. EGFR expression depended on percentage cell growth in culture. The study thus suggests that PCNA could be used as a marker in cell culturing especially when the latter would serve as tool for clinical diagnosis or other investigations.

Key words: McCoy-Plovdiv, Ki67, PCNA, EGFR, Proliferation

INTRODUCTION

In vitro cell culturing techniques provide a wide variety of scientific approaches in fundamental, diagnostic and therapeutic fields of biomedical research. The development of modern biotechnologies, experimental and applied immunology, pharmacology and gene therapy would be inconceivable without the application of cell culture systems (1). Serum-free cell lines are specific, technologically precise and cost-effective substrates for testing biologically active substances and detecting different pathogens (2)

The McCoy-Plovdiv cell line is a new cell line produced in our laboratory. It was derived from the standard serum-supplemented synovial cell line McCoy (3). It is an immortalized fibroblast cell culture adapted to growth and culturing in serum-free culture medium. The cell line McCoy-Plovdiv has several advantages some of which are the following: cells are protected

from stress due to changes in medium composition, variations between different serum batches, contamination and interactions between test substances and serum components. This new serum-free cell line is approximately 20 times cheaper than the standard serum-supplemented cell lines (4). All these then call for detailed studies on the proliferative characteristics of the McCoy-Plovdiv cell line.

Studies on the expression of specific proliferative molecules such as Ki67, PCNA and EGFR in serum-free cell lines have been quite limited and none has been done using this new cell line.

PCNA (proliferating cell nuclear antigen) is one of the vital molecules responsible for DNA-replication and repair. Its expression is regulated at both transcriptional and posttranscriptional levels (5). The PCNA gene is induced by p53. Hence in non-dividing cells the protein is present in low concentration. But in the G1, however, activation by cdk2 phosphorylation triggers its synthesis (6). The protein plays a role in DNA synthesis by acting as a co-factor for DNA-polymerase- δ (7).

Ki67, on its part, is a commonly

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applied marker for assessing cell proliferative activity and is used to calculate the so-called proliferating index, which is a ratio of dividing/non-dividing cells. It is a large protein of approximately 395 kDa. Ki67 appears in mid-to-late G1-phase and increases as the cell progresses through the S-phase to reach maximum expression at the G2-phase. Then it falls to zero in resting (G0) cells (8). Ki67 is located in the nucleus but relocates to another site during mitosis. It is a DNA-binding protein affecting chromatin structure and function (9). Results of recent studies have suggested that the Ki67 protein was involved in the protein interaction network that drives cell division cycles, possibly by interacting with phosphorylated proteins. This notwithstanding, the exact function of Ki67 is still unclear. In any case, it is essential for cell proliferation, since its removal by antisense nucleotides prevents cell growth (10).

The epidermal growth factor receptor (EGFR) contains a cytoplasmic tyrosine domain, a single transmembrane domain and an extracellular domain that is involved in ligand binding and receptor dimerization (11). Phosphorylation creates binding sites for interaction with different enzymes and adaptor molecules. These interactions are critical for the induction of those signaling events regulated by EGFR (12). EGFR is detected in the nucleus in many tissues and cell lines. Recently it was shown that EGFR associated with the promoter region of cyclin D1, a protein playing a key role in the cell cycle. This result suggests that EGFR might function as a transcription factor (13). No doubt then, the use of these cell-cycle-related proteins could help in any study associated with cell proliferation.

Therefore the aim of the present study was to examine the proliferative potential of the new serum-free cell line McCoy-Plovdiv by investigating the expression profiles of Ki67, PCNA and EGFR.

MATERIALS AND METHODS

The serum-free cell line McCoy-Plovdiv was isolated and successfully stabilized at the Laboratory of Cell Biology (University of Plovdiv) (3). Cell cultures were maintained over time by subculturing every 6-8 days. They were grown in Ham's F-12 (Serva) and DMEM (Serva) media combination of ratio 1:1. The resulting culture medium was supplemented with vitamins, L-glutamine and antibiotics (all from Sigma).

The cells were cultured for the following times in hours: 1, 6, 24, 48, 72, and 96 and from Day 6 through 17. At the end of each culture period the cells were fixed with ice-cold 80% solution of acetone for 5 minutes. Endogenous peroxidase was blocked with a 5% solution of H₂O₂. To determine PCNA, Ki67 and EGFR expressions monoclonal antibodies to human EGFR, Ki67 and PCNA (DAKO) were used as primary antibodies. The immunocytochemical reaction was visualized using 2% solution of aminoethylcarbazole as substrate in the Biotin-Streptavidin-Peroxidase labeling system (DAKO). EGFR expression was investigated on subconfluent and confluent cultures. The reddish-brown granular accumulations were termed positive reaction. The nuclei were counterstained with Mayer hematoxylin.

RESULTS

PCNA was detected under the nuclear membrane on the 1st hour of McCoy-Plovdiv cell culturing. The expression peaked on the 72nd hour, when the whole nucleus and the perinuclear zone stained positive (**Figure 1a**, **Figure 1b**). Then the expression declined after Day 8.

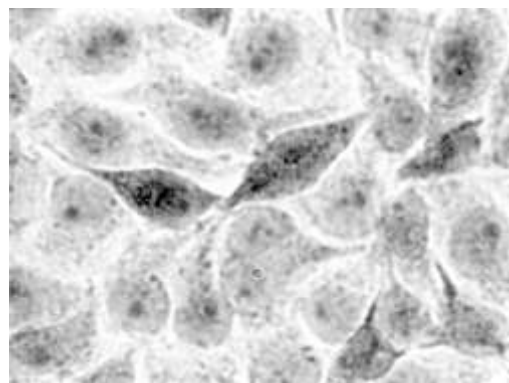


Figure 1a. Expression of PCNA on the 72nd hour of culturing of serum-free cell line McCoy-Plovdiv (x 400)

Only the nucleoli stained positive for Ki67 on 24th hour. From 72nd through 96th hour a diffuse positive reaction in the whole nucleus was observed. Ki67 staining was also seen in the cytoplasm (**Figure 2a**, **Figure 2b**). Ki67 expression declined after Day 6.

EGFR was expressed by the whole cell population in subconfluent cultures, notably on their plasma membranes, as well as in the cytoplasm and the nuclei. In confluent cultures, however, the intensity of the expression and the number of EGFR positive cells strongly declined.

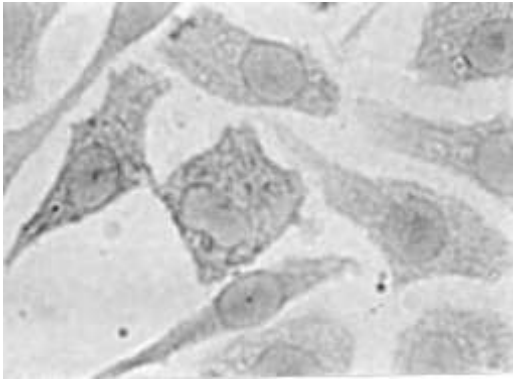


Figure 1b. Expression of PCNA on the 96th hour of culturing of serum-free cell line McCoy-Plovdiv (x 400)

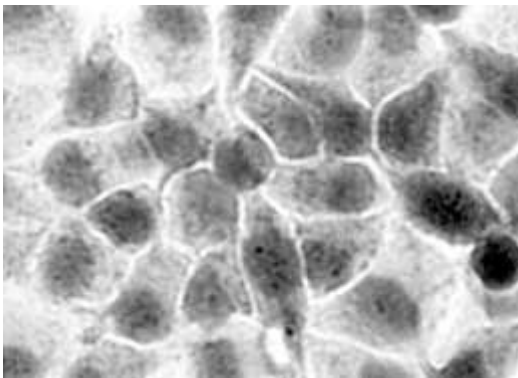


Figure 2a. Expression of Ki67 on the 72nd hour of culturing of serum-free cell line McCoy-Plovdiv (x 400)

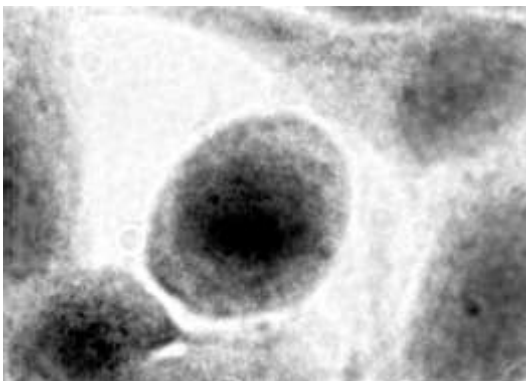


Figure 2b. Expression of Ki67 on the 96th hour of culturing of serum-free cell line McCoy-Plovdiv (x 1000)

DISCUSSION.

The proliferative activity of the new serum-free cell line McCoy-Plovdiv, using monoclonal antibodies to specific proliferative antigens PCNA, Ki67 and EGFR in an immunocytochemical study, was examined. These markers are often used to assess the proliferative status of tissues. And not quite long ago serum-free cell lines have been gaining popularity as a test-system in the detection of different pathogens and biological products that could affect elements

of the cell regulatory machinery and therefore cell proliferation. This then underscores the need for more elucidative and informative laboratory methods of assessing cell proliferative activities. In pursuance of this goal our study has proposed a model test-system based on the new serum-free cell line McCoy-Plovdiv.

The results from our study have shown PCNA as an early and informative marker of the proliferative activity of the new serum-free cell line McCoy-Plovdiv. It was expressed on the 1st hour and persisted till Day 8 of culturing. Previous studies have shown PCNA present in G0 cells in which it was inactivated by p21 (14).

The appearance of Ki67 on 24th hour of our study with the McCoy-Plovdiv culture setup could indicate cell cycle progression to the S-phase. The period of Ki67-negativity could mean a time when cells were conditioning to the culture medium and at which time they could have been under the G0- or early G1-phase arrest. The 72nd hour of culturing showed both PCNA and Ki67 expressions as diffuse stain reactions. This period could have portended some active DNA synthesis. The result with Ki67 could mean that it alone may not serve as a sufficiently informative marker owing to its relatively later time of expression. The cytoplasmic staining for Ki67 might be due to some aberrant synthesis or to nuclear membrane rupture during mitosis.

It could be speculated at this stage that some biological substances could alter the expression of Ki67 in an *in vitro* testing for anti-proliferative effects. However, using a parallel investigation with both markers, Ki67 and PCNA in a McCoy-Plovdiv cell line setup, any potential errors could be eliminated.

Growth factors such as IGF-1 increase PCNA expression in cultured cells (15). The same effect might be expected if McCoy-Plovdiv cells were treated with potential carcinogenic products or growth-promoting substances. On this score one might expect some increase in Ki67 expression as well. On the other hand, cytotoxic lesions are repaired by a PCNA-dependent pathway and high levels of this protein could be the cells' antidote against cytotoxic agents. Such a situation could explain the PCNA elevation after the 24th hour of culturing or after Ki67 expression (that is, following the transition from S- to G2-phase of the cell cycle) in the McCoy-Plovdiv cell line setup.

EGFR was highly expressed in

subconfluent cultures. This was characterized by a rising trend to a peak and then a trailing off to a decline. One would suggest the existence of an EGFR pathway involved in the control of McCoy-Plovdiv cell growth. The significant reduction of EGFR presence, as shown by low staining reactions, in confluent cultures would suggest that this receptor might play a role in cell adhesion and in contact inhibition. As EGFR was shown expressed on the surface of McCoy-Plovdiv cells it could possibly affect intercellular interactions and cell culture maintenance. Further studies are required to establish the precise cellular localization and time of appearance of EGFR in McCoy-Plovdiv cells.

Serum-free cell lines are preferable

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over others in biomedical and pharmacological studies for reasons so far adduced. In view of this the McCoy-Plovdiv has been used as a test system for examining the effect of different substances (4). And because of the attributes shown so far by this system in our present study we propose that PCNA alone could be used as a marker in the culture system where it could have diagnostic and other investigative potentials.

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