Original Article

LABELLING TRANSFORMED MITOTIC CELLS WITH FLUORESCENT ANTIBODY CONJUGATE

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ABSTRACT

The functions of most living cells are characterized by well-governed events that encompass the cell cycle. Cell division primarily occurs as mitosis, which is regulated and follows a defined series of mechanisms. A key player in regulating mitosis is a group of proteins collectively called the mitotic factors, which are initially localized in the cytoplasm. These appear gradually and peak at the mitotic stage of cell cycle. Protein profile analysis of interphase and mitotic cells show distinct differences and this was utilized for the premature condensation of interphase DNA through techniques involving somatic cell hybridizations and mitotic extracts. Studies of cell cycle kinetics and the underlying mechanisms have become possible with the continuous cultures of cell lines in vitro. We have raised polyclonal antibodies to the mitotic proteins of Chinese Hamster Ovary cells and the predominantly IgG antibodies from the whole serum were purified following induction of a secondary immune response. The purified IgG was conjugated to FITC, a fluorescent dye and the conjugate was used to stain interphase and mitotic cells of immortalized cells in culture and also cultured peripheral blood human lymphocytes. It was observed that the conjugate selectively stained immortalized mitotic cells either in suspensions or in preserved tissue sections. While the cross reactivity with immortalized mitotic cells of varied types was evident, the non-reactivity to untransformed cells gives us a means of further utilizing these antibodies to potential applications such as in cancerous conditions where cell transformation and uncontrolled mitotic events are the underlying factors.

Key words: *Chinese hamster ovary cell line, polyclonal antibodies, mitotic factors, immunofluorescence.*

INTRODUCTION:

Cell growth and division comprises a series of discrete stages, collectively known as the cell cycle (1). The cell cycle begins when the division of a single parental cell forms two new cells and ends when one of these cells divides again into two cells. This division process, called the "M phase", involves two overlapping events, the karyokinesis followed by cytokinesis (2). Although the cells of a multicellular organism divide at varying rates, most studies of the cell cycle involve cells growing in culture where the length of the cycle tends to be similar for different cell types. Determining the overall length of the cell cycle - the *generation time* – for cultured cells has been achieved and reported earlier (3). For mammalian cells in culture, S phase is about 6- 8 hours in length. Similarly, estimating the length of M phase by multiplying the generation time by the percentage of the cells that are actually in mitosis at any given time is possible. This percentage is called the *Mitotic index*. The mitotic index for cultured mammalian cells is often about 3-5 %, which means that M phase lasts less than an hour (usually 30 -45 minutes) (4).

The cell cycle control system is based on two key families of proteins. The first is the family of *cyclin dependent protein kinases (Cdk),* which induce downstream processes by phosphorylating selected proteins on serines and threonines. The second is a family of specialized activating proteins, called *cyclins* that bind to Cdk molecules and control their ability to phosphorylate appropriate target proteins. The cyclin assembly, activation and disassembly of cyclin-Cdk complexes are the pivotal events driving the cell cycle (3). Cyclins undergo a cycle of synthesis and degradation in each division of the cycle of the cell. There are two main classes of cyclins: *mitotic cyclins*, which bind to Cdk molecules during G2 and are required for entry into mitosis, and *G1 cyclins*, which bind to Cdk molecules during G1 and are required for entry into S phase.

Cell fusion experiments suggested that specific molecules present in the cytoplasm are responsible for moving the cells through the G1 and G2 checkpoints (that is, for triggering the onset of DNA replications phase) and mitosis (M phase) (5). Evidence regarding the mitosis-triggering signal has come from experiments involving frog eggs. During development of the frog oocyte (an egg cell precursor) the cell cycle is arrested in G2 until hormones stimulate meiosis. The oocyte then proceeds through most of the phases of meiosis but is arrested during metaphase of the second of two meiotic divisions. If cytoplasm taken from a mature egg cell is injected into the cytoplasm of an immature oocyte, the oocyte immediately begins meiosis (6). It was hypothesized that a cytoplasmic molecule, which were named maturation promoting factor (MPF), induces this oocyte "maturation".

Subsequent experiments revealed that in addition to inducing meiosis, MPF can also trigger mitosis when injected into fertilized frog eggs (6). Similar MPF molecules were later discovered in the cytoplasms of a broad range of dividing cell types, including yeast, marine invertebrates, and mammals. Because of the general role played by MPF in triggering passage through the G2 checkpoint and into mitosis, MPF, which originally stood for "maturation promoting factor" is now used to mean "mitosis promoting factor", a term that describes this molecule's role.

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As distinct protein profiles are evident in the interphase and mitotic phases of the cell cycle, we utilized the antigenic properties of proteins present in the mitotic stage to raise polyclonal antibodies against the same. Antibodies being very specific in their nature of activity, we attempt to demonstrate the specific reactivity of these antibodies to cells at the mitotic stage of transformed cells. Immunofluorescent techniques provide us with the best possible visualization in cytological applications and therefore we used FITC conjugated antibodies to demonstrate the localization and reactivity to varied cell types in both interphase and the mitotic stages.

MATERIALS AND METHODS:

Polyclonal antibodies to CHO mitotic cytosolic proteins:

A continuous culture of CHO cell line was maintained according to standard protocol. Briefly, the cultures were maintained in DMEM with 5% serum supplementation at 37^0 C under 5% humidified atmosphere (Fig1).

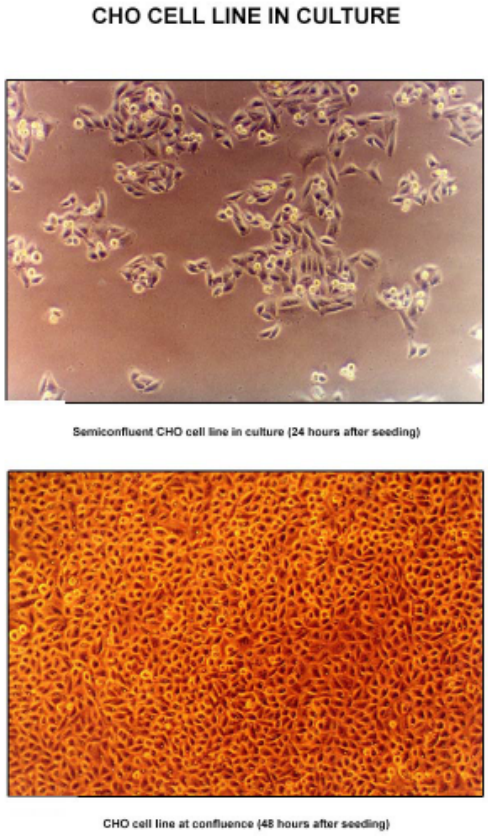


Figure 1: *CHO cells in culture. Typically, cells show attachment in a few hours after passaging and attain healthy confluence in about 48 hours in a T-25 flask. Culture conditions are in DMEM supplemented with 5% FBS; 37^0 C with 5% CO_2.*

Mitotic CHO cells were harvested and the cytosolic proteins extracted as previously described. Rabbit polyclonal antibodies were raised against the extracted mitotic cytosolic proteins and identified IgG as the predominant isotype further to the induction of secondary immune response (7). IgG was affinity purified from whole serum, concentrated to obtain a final titre of 1:8 and was used for the FITC conjugation. The chromatogram of IgG affinity purification is given in Fig 2.

FITC conjugation to Anti-CHO mitotic protein antibodies

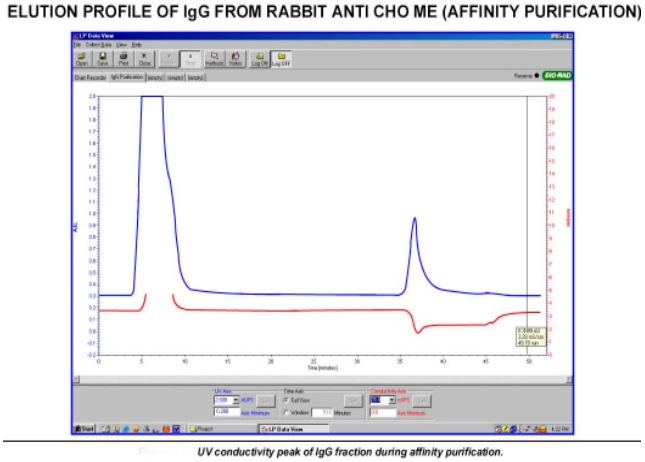


Figure 2: *Chromatogram of IgG purification from rabbit anti CHO mitotic cytosolic protein whole serum. Further to the secondary booster, the predominant antibody Isotype was IgG as determined by Ouchterlony Double Diffusion. IgG was purified to avoid the usage of whole serum to minimize non-specific interactions.*

Fluorescein isothiocyanate (1.0 mg) was reconstituted with 200μL of the solvent (sterile water or 40-50% glycerol). 250 μL of affinity purified IgG fraction (after concentration) was mixed with 1/10th volume (25 μL) of sodium carbonate bi carbonate buffer [7.5 mL of 0.2 mol/L sodium carbonate solution diluted with 42.5 mL of 0.2 mol/L sodium bicarbonate solution to 200 mL with distilled water]. 15 μL of reconstituted FITC solution was added to the above and incubated at room temperature for 2 hours on a shaker in dark. Following incubation, 1/20th volume (30 μL) of 1M ammonium chloride was added and incubated at room temperature for 1 hour on a shaker in dark. Sephadex G-25 column was washed with 20 mL 1X PBS to obtain base line UV and conductivity levels. 300μL of reaction mixture (IgG labeled with FITC) was loaded to the bed of the column and eluted through the column using 1X PBS as the wash buffer. The elution profile was recorded and the first fraction (containing the IgG-FITC conjugate) was collected. The IgG-FITC conjugate was stored at 4°C with 1%(w/v) BSA and 0.1% sodium azide.

Target cell preparation and immunostaining:

While interphase cells of the cell lines in culture (CHO, Vero and HeLa) remain attached to the culture flasks, those in the mitotic phase round off and detach from the culture flasks (Fig 3). Mitotic cells were collected from cell lines by gentle mechanical agitation from confluent flasks and were individually processed by washing (1000 rpm; 10minutes) thrice in 1X PBS.

A 72-hour human lymphocyte (whole blood) culture was set up according to conventional cytogenetic

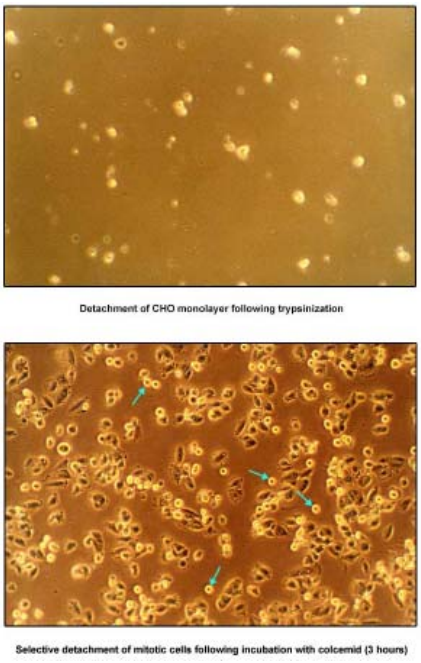
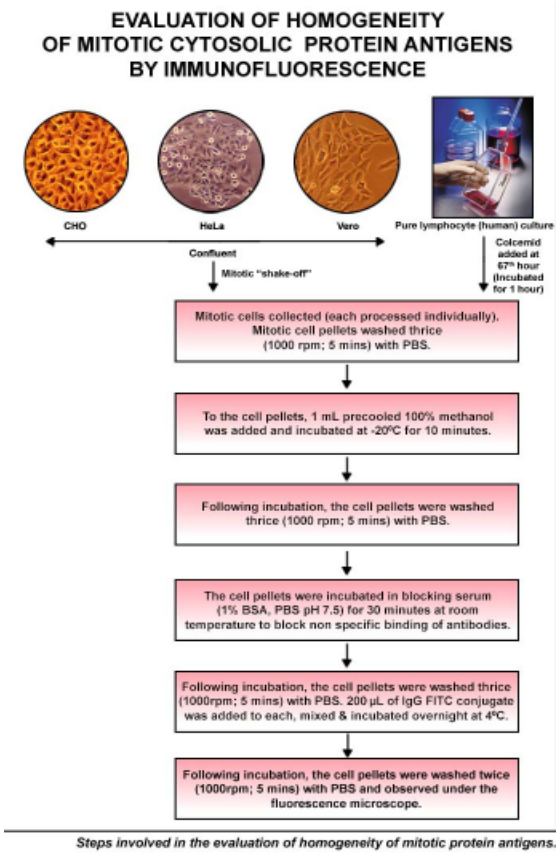


Figure 3: *CHO Interphase and Mitotic cells; upon trypsinization, cells detach from culture flask enabling passaging and maintenance of the cell line. In a healthy culture, Interphase cells remain attached to the flask with distinct spindle shaped morphology and the Mitotic cells round-off and detach from the flask surface; thus enabling selective harvest of cells at the Mitotic stage.*



Flowchart 1: Protocols employed for obtaining target cells and their processing for immunofluorescent staining.

procedures. The culture was initiated in RPMI 1640 medium with 10%FCS and incubated at 37°C, 5% CO_2. Phytohaemagglutinin was added to stimulate lymphocyte proliferation in vitro.100μL of 0.01% Colchicine (mitotic blocker) was added at the end of 70 hours and incubated for 90 minutes; following which the cells were harvested by a brief exposure (20 minutes) to hypotonic solution (0.075M pre warmed Potassium Chloride), centrifuged at 1000 rpm for 10 minutes and the cell pellet was collected.

The cell pellets thus collected of the various cell types employed for this study were incubated in 150 μL blocking buffer for 30 minutes at room temperature and following incubation they were washed twice in 1X PBS and the cell pellets were resuspended in minimum quantity of 1X PBS. To each of the cell pellets 200 μl of IgG-FITC conjugate was added and incubated at 4°C overnight. Following overnight incubation the cells were washed (1000 rpm; 10 minutes) in 1X PBS and layered on glass slides and observed under the fluorescence microscope (Flow chart 1).

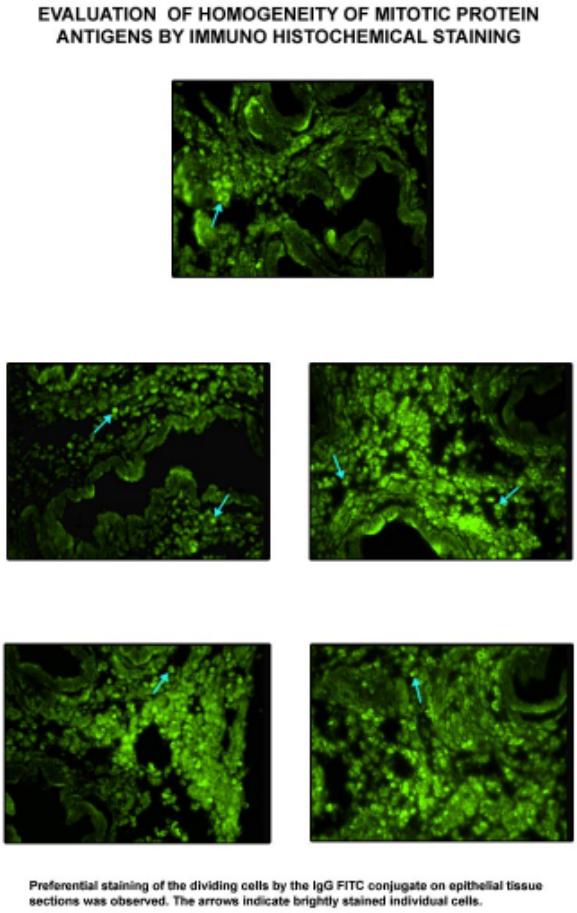


Figure 4: *Immunofluorescent staining of epithelial tissue section by the Anti CHO mitotic cytosolic proteins IgG-FITC conjugate revealing selective staining of cells.*

Epithelial tissue that was paraffin embedded and preserved was washed in 1X PBS for 5 minutes three times. The section was rehydrated in alcohol series (95%, 90%, 85% ethanol) for 2 minutes in each three times. The slide

was rinsed in 1X PBS for 5 minutes twice. The section was incubated in blocking serum for 15 minutes. Following incubation 250μL of IgG-FITC conjugate was added to it and the slide was incubated at 4°C overnight in a humid chamber. Following overnight incubation, the slide was rinsed once with 1X PBS and observed under the fluorescence microscope.

RESULTS:

Concentrated IgG fraction (250μL) was conjugated with FITC and purified by desalting to remove unbound dye from the conjugate. A final volume of 2 ml of the purified IgG-FITC conjugate was collected.

A mixture of interphase and mitotic cells from CHO, Vero, HeLa and human peripheral blood lymphocytes harvested and stained with IgG –FITC conjugate demonstrated selective staining of mitotic cells. The same was observed on paraffin embedded epithelial tissue sections; only a few cells in the tissue were preferentially stained by the IgG-FITC conjugate (Fig 4).

DISCUSSION:

Mitotic cells of human origin (HeLa), upon fusion, can induce Premature Chromosome Condensation in cells from a variety of animal species including mammals, birds, amphibians, fishes, and insects, and mitotic cells from these species can induce PCC in HeLa cells indicates that the factors involved in the induction of this phenomenon are common over a wide range of animal species. In fact plant cells have been shown to induce PCC in mammalian cells. The factors involved are most likely proteins since, if the mitotic inducer cells are prelabeled with radioactively tagged aminoacids, labeled protein can be observed to be transferred from the mitotic component to the interphase chromosomes (8, 9).

The mitotic factors being proteins biochemically, their antigenic properties can be fairly well exploited and suitable antibodies produced that can specifically react with the same. Such antibodies, moreover if polyclonal in nature, by virtue of the affinity to antigenic epitopes can be useful tools for evaluating homogeneity of mitotic cytosolic protein antigens. Also, the cross reactivity of the antibodies to structurally similar antigenic epitopes might prove to be valuable in comparing soluble proteins. Immunofluorescent and immunohistochemical techniques employing suitable antibody conjugates become useful detection tools for specific cell populations or those that express unique antigenic epitopes.

The cross reactivity of polyclonal rabbit Anti CHO mitotic extract with mitotic cells of CHO, Vero, HeLa and normal human lymphocytes was earlier demonstrated to evaluate the homogeneity of the cytosolic protein antigens (10). The results demonstrated the reactivity of the whole serum and IgG fraction only to the mitotic extracts from immortalized cell lines (CHO, Vero and HeLa) and failed to react with the protein extracts from normal human lymphocytes indicating the presence of unique protein or antigenic epitope in mitotic extracts of cell lines with altered cell cycle kinetics which is not present in normal cells (human lymphocytes). It was also inferred that the reactivity of the antiserum is strongly influenced by the presence of this particular protein or the epitope.

CONCLUSION:

The fluorescent antibody conjugate employed in this study was effective in detection and selective labeling of immortalized mitotic cells from a mixed population either in suspension or in preserved tissue section. This while demonstrating the presence of shared epitopes in altered cell cycle conditions, also gives us the possibility of exploitation of these antibodies for a variety of applications especially in cancerous situations where, the breakdown of the mitotic machinery is the hall mark that results in unregulated mitotic proliferation of cells.

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