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Construction of High Expression Vector System Producing Therapeutic Monoclonal Antibody Through Incorporation of Matrix Attachment Region Element: A Preliminary Report

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Abstract: The emergence of monoclonal antibody specific to human growth factor receptor marked the greatest achievement in human fight against cancer. By utilizing a specific antibody, cancer treatment nowadays is getting more specific and efficient. Despite its achievement, the main drawback of antibody therapy remains at its production level. Low yield and transient expression of antibody by mammalian host cells are among the problems discovered during antibody production. Many strategies have been implied to increase yield and stability of protein including improvement of vector expression system with DNA elements integration as well as manipulating the bioreactor environment to increase cell density and attain more products. Our study is currently focused on constructing a high expression vector system using matrix attachment region (MAR) element in targeted therapeutic monoclonal antibody production. In this study, a nuclear halo formation has been successfully optimized for CHO cells. A minimum time of 8 minutes is required for maximum nuclear halo formation, thus exposing the most significant sites containing MAR element in CHO genome. This preliminary step is crucial due to the fact that the nuclear halo formation and MAR elements isolation are specific for different types of cells. Subsequent works are currently being carried out to isolate the MAR element from CHO cells based on the time determined from nuclear halo formation. This work is going to be distinctive from previous studies as the MAR element will be predicted from the host cells genome. The mechanism of which the integrated functional MAR element can increase the antibody production even at random position is through up-regulation of gene transcription by adopting a DNA loop structure of nucleosomes that open the structure for specific transcription factor binding.

Keywords: Monoclonal antibody, Matrix attachment region, Mammalian cell culture.

INTRODUCTION

Monoclonal antibody therapy against cancer is considered as one of the best approaches to overcome this disease at any stage of incidence. With post-genomic era on the rise, most carcinogenic pathway has been out ruled and understood. By understanding the biology of cancer cells, researchers are now able to identify the exact target and thus minimize the toxic effect of cancer therapy. Using monoclonal antibody against specific antigen, elimination of cancer cells now depends on the immune system of patients. Herceptin (Trastuzumab) is a humanized monoclonal antibody drug designed against human HER2 receptor tyrosine kinase. HER2 is also a member of the epidermal growth factor receptor family of transmembrane receptor [1]. Herceptin was approved by Food Drug Administration (FDA) as a drug for metastatic breast cancer in 1998. Since then, this drug has proved to be useful in combination with chemotherapy [2, 3]. Despite its high efficiency against HER2 positive cancer, the use of herceptin treatment is hindered by its high cost. The full course of herceptin treatment for a single patient has been reported to cost about USD 70,000 [4]. This fact, limits the overall survival of breast cancer patients due to unaffordable high cost treatment.

Despite being specific and efficient in fighting cancer, the main drawback of antibody therapy remains at its production level. Low yield and transient expression of antibody by host cells are among the problems discovered during antibody production. This is due to the fact that introduction of foreign genes into other genomes is usually followed by random integration at various chromosomal sites. Therefore, optimizing the foreign genes at the construction level has become the best approach to ensure high degree of protein production. Approaches such as DNA elements integration [5], promoter element integration [6] and scaffold/matrix attachment region integration [7] into expression vector system have proved to be effective. Another factor that has significant effect towards recombinant protein production and has been applied extensively is insertion of signal sequence coding region together with transgene

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sequence which facilitates in mRNA export to reticulum endothelium for processing [8]. Among the strategies applied the current popular approaches involve intrinsic modification like incorporation of DNA element such as matrix attachment regions (MAR) into expression vector to facilitate the transgene expression by maintaining the open chromatin structure during the transcription process [9].

MARs are 70% AT-rich region with 300 to 3000 bp long DNA elements which is similar to topoisomerase II consensus sequence that were proposed to attach chromatin to proteins of the nuclear matrix [10, 11]. Several reports have proposed that by flanking transgene sequence with MAR element, certain regions of chromatin will attach to the nuclear matrix resulting in loop formation that acts as a boundary for the transcriptional machinery to initiate transcription of the inserted transgene [18, 19]. This will hinder specific regions from silencing effects, therefore increasing the chance of genes being transcribed. Furthermore, MARs have been proved to be co-localized with transcription regulatory element which further facilitates in both transcription and replication [12]. Previous studies have shown that incorporation of MAR elements in any configuration has affected the level of protein being expressed. This, however, depends on the location of MAR integration inside the expression vector or co-transfection of MAR into host cells [9, 13]. The most favorable locus for introducing MAR is proposed to be upstream of promoter and enhancer region in expression vector [9]. Even the proper mechanisms by which MAR elements contribute to increase protein being expressed still remain unclear, several studies have reported on the enhancement of transgene expression with copy number-dependent or position-independent properties of MAR elements [7, 14, 15]. Recent finding has reported that combination of MAR element with mammalian replication initiation region in CHO DG44 cells significantly initiated gene amplification effectively and spontaneously increased gene copy number [16].

In this study, we are aiming on constructing a high expression vector system using MAR elements predicted and isolated from our own CHO genome database. MAR elements will also be isolated from CHO cells and sequenced for analysis. Vector construction will be carried out to express anti-her2 monoclonal antibody in CHO cells and protein production will be measured and compared. This study is still in preliminary stage; therefore, only relevant results will be discussed.

MATERIALS AND METHODS

Cell Culture and Maintenance

CHO (DG44) cells in suspension retrieved from industrial partner, Inno Biologics (M) Sdn. Bhd. were cultured in SFM4CHO™ (HyClone, USA) media in spinner flask at 37°C and 5% CO_2 insulation. The medium is supplemented with 4 mM L-glutamine and changed every 3 to 4 days.

Preparation of CHO Cell Nuclear Matrix for Nuclear Halos Formation on Slides

Experiment was conducted based on the previous method published by Krawetz *et al*. [17] with some modifications. Briefly, CHO cells were grown in serum-free suspensions and were harvested during their log growth phase with viability of more than 90%. Approximately 1.5 x 10^6 cells/mL cells were used as starting material. The medium was removed and cells were collected and washed in phosphate buffered saline (PBS) containing bovine serum albumin (BSA) and proteinase inhibitor. Cell pellets were suspended in nuclei buffer and incubated on ice for 15 minutes. A total of 100 µL cell suspension was subjected to cytospin (Hettich Zettrifuge, German) and attached to clean glass slides for DAPI staining. Equal volumes of cell suspensions were embedded in gel prior to attachment on slides for ethidium bromide staining. Histone extraction was performed by overlaying the attached cells with 2 M NaCl at different time intervals. Reaction at different time points was stopped by washing and dehydrating the cells in serial diluted ice-cold PBS (10X, 5X, 2X, 1X) and ethanol baths (50%, 70%, 95%, 100%). Cells were fixed by baking at 70°C for 2 hours and preceded for image analysis using fluorescence microscope (Olympus America Inc., USA).

Analysis of Nuclear Halo Formation

The pre-processed CHO cells were incubated with 1X DAPI and 20 µg/mL ethidium bromide for 1 minute in dark prior to visualization under fluorescence microscope imager. Each slide representing a different time point was analyzed for nuclear halo image and the distance from extracted DNA perimeter to the edge of nuclear matrix was measured. The minimum time required to reach maximum halo diameter is considered optimal [17] for nuclear halo in solution preparation. The experiments were performed in triplicate to ensure data integrity.

Synthesis of anti-her2 Variables for Vector Construction

Nucleotide sequences of variable light (VL) and variable heavy (VH) domain of herceptin were retrieved from Drug Target Database and subjected to synthesis. A cDNA clone of anti-her2 variables for heavy and light chain was retrieved from industrial partner in plasmid form and subjected for transformation. Plasmids extraction was performed by alkaline lysis and analyzed by gel electrophoresis. A specific pair of primers for anti-her2 variables heavy and light chains were designed to introduce a specific restriction site and signal sequences into the transgene cassettes. A PCR was performed for both heavy and light chain variables and products were analyzed and visualized by gel electrophoresis.

RESULTS

Nuclear Halo Formation in CHO cell

A nuclear halo feature was observed in CHO cell as a fluorescent ring around cell nucleus (Fig. **1**). Exposure to 2M NaCl at different time intervals resulted in a different size of halo ring as it has been shown by DAPI (Fig. **1**) and ethidium bromide (Fig. **2**) staining. The distance from extracted DNA perimeters towards nuclear matrix edges was measured at each time point and optimum time for halo formation was determined 8 minutes (Fig. **3**). The optimum time point was then applied in nuclear halos preparation in solution for MAR extraction (data are not present).

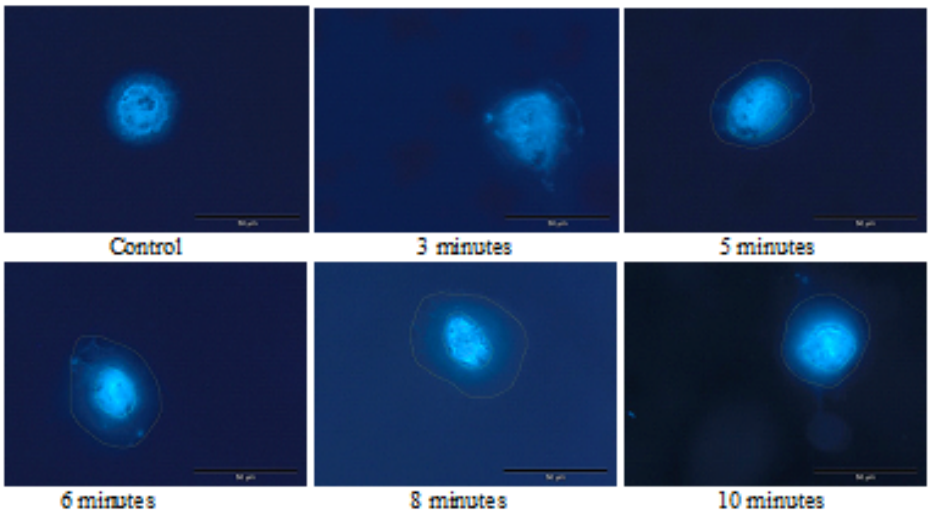

Fig. (1). Nuclear halo formation in CHO cells visualized with 1X DAPI staining.

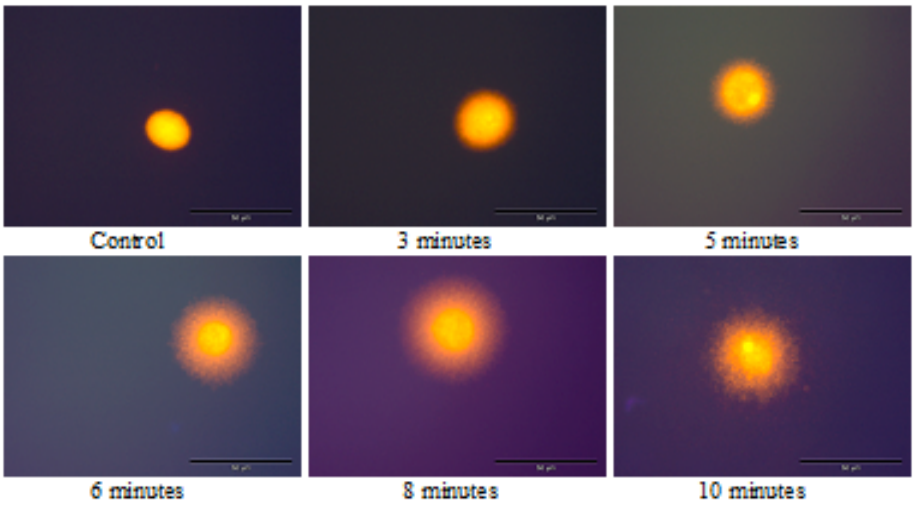

Fig. (2). Nuclear halo formation in CHO cells visualized with 20 µg/mL ethidium bromide.

Synthesis of anti-her2 Variables for Heavy and Light Chain

At the same time, the anti-her2 variables region for heavy and light chain were prepared and synthesized from pIDTSMART (Integrated DNA Technologies, USA). Plasmid extraction of cDNA clones containing anti-her2 variables for heavy and light chain resulted in plasmid band 2300 to 2400 bp in sizes (Fig. **4A**). PCR was performed and optimized using specific primer pairs towards anti-her2 variables for heavy and light chain. Reactions were optimized to amplify both sequences and resulted in specific products at 300 to 400 bp in sizes (Fig. **4B**). Specific PCR product for each clone is achieved by proper primer designing with high Tm° value of more than 65°C and high final concentration of $MgCl_2$ (3.2 mM per reaction). However, plasmid backbone is still traceable due to high copy number of cloning vector. Purified PCR products are sent for sequencing for sequence identification (data not shown).

DISCUSSION

The nuclear halo represents the loop that formed upon attachment of MAR to the nuclear matrix. Generally, isolation of MAR is performed by extracting cell nuclei followed by histone removal to allow the formation of loop that formed as a result from the anchoring of MAR to the nuclear matrix. Restriction digestion will then remove the residual genomic DNA leaving only MAR that binds to the nuclear matrix [19]. However, the signal which induces MAR to bind to nuclear matrix remains unclear. By using the 2 M NaCl method with some modifications, we are able to demonstrate the nuclear halo formation inside the CHO cells. This step was performed to determine the minimum time required for maximum halo span associated with optimal time for nuclear protein extraction [17]. The use of 2 M NaCl on permeabilized cells resulted in histones released and chromatin been unwind into loops of different sizes. MAR elements are proposed to locate at the base of nuclear matrix and play an important function in segregating the chromatin into distinct independent loops, thus provide a protection for genes inside the loops from silencing effect [9]. Our study has proved that the intact nuclear halo images without noise in CHO cells are retrievable without complex intervention during cells processing where a simple additional step was introduced at the beginning of the experiment. By embedding CHO cells in the agarose gel, assessment on nuclei changes was easily performed. These

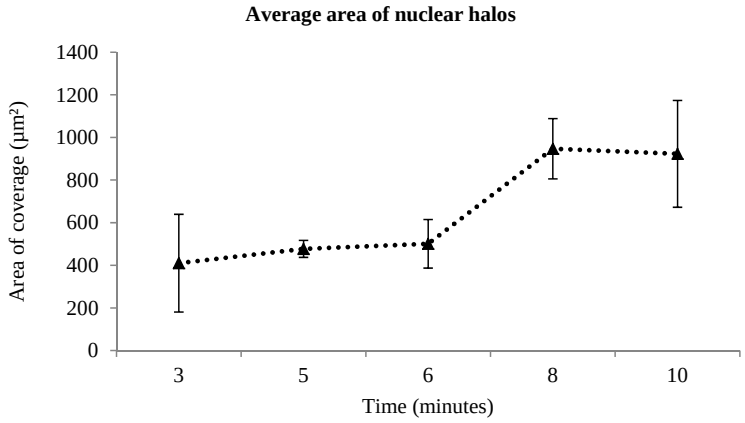

Fig. (3). Line graph showing a different size of nuclear halo area at different time points.

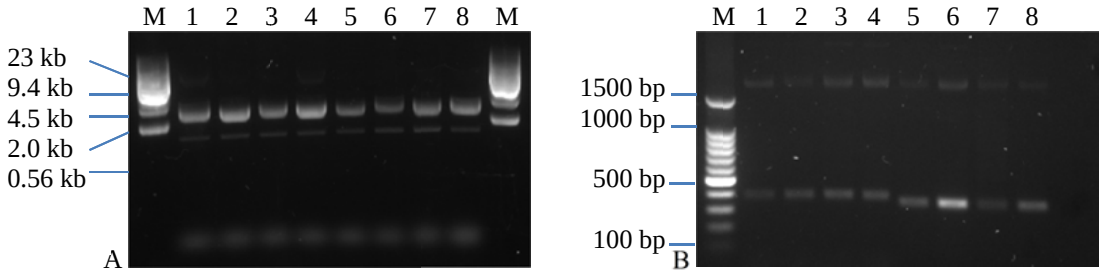

Fig. (4A),. Uncut plasmids containing anti-her2 variables. (M, supercoiled DNA marker; Lane 1-4, plasmids with heavy chain variables; Lane 5-8, plasmids with light chain variables). **B**. PCR products of anti-her2 variables. (M, 100 bp DNA marker; Lane 1-4, heavy chain variables (375 bp); Lane 5-8, light chain variables (333 bp)).

techniques also provide a cleaner background which is usually introduced by cells artifact upon harsh processing. A total of 8 minutes are needed for CHO cells chromatin to reach farthest distance from nuclear matrix edge before starting to recline. Thus, 8 minutes is the minimum time required by CHO cells chromatin to reach maximum loops formation and expose the MAR elements attached to its nuclear matrix. The same principle will be applied in solution where loops formed after 8 minutes will be digested, leaving the MAR elements remain attached to the nuclear matrix. The MAR element will then be separated from nuclear matrix by protein digestion using proteinase K. The isolated MAR elements will be cloned, sequenced and analyzed against our own CHO genome database prior to insertion in the expression vector.

CONCLUSION

Nuclear halo formation of CHO cells has been successfully optimized and protocol for cleaner background has been properly developed. This work is still in preliminary stage and further experiments to isolate MAR elements and cloning of anti-her2 variables into expression vector for heavy and light chain are currently being carried out.

CONFLICT OF INTEREST

All authors listed above have contributed to the manuscript's preparation and experimental works performed to retrieve the data. The cDNA clones of anti-her2 variables for heavy and light chain are a courtesy of our industrial partners; Inno Biologics (M) Sdn. Bhd. Materials supplied by the industry are restricted to research works only.

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