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(54) Titre : METHODE PERMETTANT D'ACCROITRE LA PRODUCTION DE CELLINE PAR L'UTILISATION D'UN PROTOCOLE DE FUSION

(54) Title: METHOD FOR OBTAINING AN INCREASED PRODUCTION OF A PRODUCING CELLINE BY USING A FUSION PROTOCOL

(57) Abrégé/Abstract:
The invention relates to a method for obtaining a higher product expression in a producing mammalian celline characterized by treating the original celline with itself according to a fusion protocol known in the art. The production of protein products is performed in mammalian tissue culture, and the product could be therapeutic proteins or other molecules intended for human use. In particular this invention relates to the use of cell fusion methods to obtain a stable high producing celline.

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(57) Abstract

The invention relates to a method for obtaining a higher product expression in a producing mammalian celline characterized by treating the original celline with itself according to a fusion protocol known in the art. The production of protein products is performed in mammalian tissue culture, and the product could be therapeutic proteins or other molecules intended for human use. In particular this invention relates to the use of cell fusion methods to obtain a stable high producing celline.

METHOD FOR OBTAINING AN INCREASED PRODUCTION OF A PRODUCING CELLINE BY USING A FUSION PROTOCOL

Field of invention

The present invention relates to a method for obtaining a higher product expression in a producing mammalian celline characterized by treating the original celline with itself according to a fusion protocol, known in the art. The production of protein products is performed in mammalian tissue culture, and the product could be therapeutic proteins or other molecules intended for human use. In particular this invention relates to the use of cell fusion methods to obtain a stable high producing celline.

Description of the Related art.

Expression of proteins in heterologous mammalian cells is usually performed in one of the following three ways, well known to the artisan in the field.

1) A vector or virus containing necessary elements for expression of proteins in mammalian cells may be introduced in an appropriate cell.
2) A primary celline expressing a certain protein may be transformed to a continuous celline.
3) A primary celline, such as a lymphocyte may be fused to a nonproducing celline and the resulting hybrid can be selected for expression of the wanted protein.

By further cultivation of cells derived from 1-3 above the protein of interest can be obtained from the culture medium or the cells themselves.

The latter technique 3 is the most common one and is used for production of monoclonal antibodies by hybridoma cells. Hybrid cellines are usually characterized by presence of chromosomes or genes from the two different fusion parents and require some sort of selection procedure in order to eliminate the parental cells from the fusion mixture and allowing the emergence and outgrowth of the fused cell.

Cell fusion as a method of enhancing protein production has been described by Chenciner et al in Bio/Technology vol 8, 1990:858-862. The authors produced classical hybrids between a liver cell and a transfected Vero cell. The stable hybrids produced an increased amount of the wanted product.

Different cell fusion protocols are well known in the art. The use of polyethylene glycol (PEG), protoplast or Sendaivirus can be mentioned as examples and reference is here given to Cell culture by William B Jakoby and Ira H Pastan, Methods in Enzymology Vol. LVIII, Academic Press, 1979. Electrofusion is another method that also can be used and reference is here given to Electromanipulation in hybridoma technology, Laboratory manual, by Carl A K Borrbaeck and Inger Hagen, Stockman Press, 1989 and Guide to Electroporation and Electrofusion, Chang et al, Academic Press, 1992. By variations of e.g. the used amount of cells, time, temperature etc., each laboratory often has its own protocol.

In summary the above described prior art discloses the use of classical cellfusion of two different cells, as means of obtaining and increasing production of a particular product in a particular cell which is a fusion cell, a combination of the two parent cells.

We have now found a method that can be used in the establishment of a protein producing celline. The method will increase the production of the wanted product of the celline.

Although the method resembles cellfusion it is not a classical cellfusion and the cellines obtained are, to our great surprise, stable over a prolonged period.

Summary of the Invention.

The present invention relates to a method of obtaining a higher product expression in a producing celline by fusing the original cell with itself according to a fusion protocol, known in the art.

In one aspect, there is described a method of obtaining a higher product expression in a producing cell line comprising fusing cells of the same origin with a fusion agent to form fused cells which produce a product.

In another aspect, there is described use of fused cells formed by the methods described herein, for producing a protein.

Cellfusion protocols suitable for the present invention are methods utilizing polyethylene glycol (PEG) or electrofusion, protoplast or Sendaivirus. The cell could be a Chinese Hamster Ovary (CHO) derived celline, Baby Hamster

Kidney (BHK) or Cos (an African green monkey derived celline) or any other commonly used mammalian celline.
The product is preferably full length factor VIII, deletion derivatives of factor VIII or otherwise modified factor VIII but could also be another therapeutically active protein or molecule.
The resulting cellines could have a chromosome and DNA content identical or very similar to the original celline.
The cellfusion protocol is preferably performed without the presence of a selection protocol, but selection protocols could be used.
The fusion protocol could be performed on already fused cells.

The invention also relates to the use of a celline or cell which has been obtained by fusing the original cell with itself according to fusion protocol in the production of a protein or another molecule and preferably a celline in which the cellines have a chromosome and DNA content similar the original celline.

The present invention comprises thus the use of a cellfusion protocol to increase the productivity of a mammalian celline.
A preferred aspect of the present invention is the use of the method to increase the productivity of human recombinant factor VIII producing cellines. The extent of increase of Factor VIII:C may be up to 2 fold, 10 fold and even 20 fold. The method is applicable to many cellines but in particular CHO (Chinese Hamster Ovary) cells producing a full length factor VIII, deletion variants or otherwise modified factor VIII.

This invention shows that under the claimed cellfusion conditions, one can obtain high producing and stable cellines which are not classical hybrids.

Description of the preferred embodiment.

Materials and Methods.
Recombinant factor VIII was produced in CHO DG44 NY cells transfected with a cDNA encoding for a deletion variant of human factor VIII called factor VIII SQ.(r-VIII SQ) (see WO 91/09122 and WO 92/16557). The following cellfusion protocol was used: The cells are cultured in a proprietary serumfree medium. 20-80x10^6 cells were washed, pelleted and a 50% (in medium) solution of PEG 4.000 was added dropwise to the pellet. After 1 minute the PEG-cell mixture was gently diluted by adding culture medium. The diluted mixture was

gently centrifuged, new medium added and further cultivated in serumfree medium. No selective medium was used and only one type of cells were used.

Fusion of the cells were monitored by chromosome counting and flow cytometry.

For measurement of factor VIII:C activity the growth medium was exchanged to a production medium and VIII:C measured as it accumulated in the supernatant (without medium changes) for the number of days indicated in the tables. Factor VIII activity (VIII:C) is measured by the chromogenic substrate (Chromogenix Mölndal) after various time points.

Example 1

This example (Table 1) illustrates the r-VIII SQ expression one week after fusion. The original r-VIII SQ producing celline "ADLA" was treated with PEG 4000 under standard fusion conditions, as described above under Materials and Methods, (ADLA PFT and PEG 1-5 in Table 1) and also when keeping the cells adhered to the plastic under subconfluency in order to prevent them from fusing (ADLA PT in Table 1). ADLA control had no PEG treatment. Cells were grown to confluence and tested for factor VIII:C.

Table 1. Expression of PEG treated cells compared to control cells (ADLA).

	Day 1	Day 2	Day 3	Day 4
ADLA Control	100%	100%	100%	100%
ADLA PT	340%	104%	123%	90%
ADLA PFT	1880%	273%	268%	184%
PEG 1	173%	97%	92%	not tested
PEG 2	236%	138%	152%	not tested
PEG 3	134%	129%	147%	not tested
PEG 4	200%	120%	159%	not tested
PEG 5	91%	85%	98%	not tested
Mean for fused	457%	140%	153%	not tested

Results

Cells treated under "standard" fusion conditions expressed as a mean 457%, 140% and 153% of control values day one to three after the fusion treatment.

Cells PEG-treated under subconfluent conditions did only express an

enhanced VIII:C activity (ADLA PT) day one after treatment. The table also shows that there are considerable differences between individual experiments and that expression is highest immediately, day one, after fusion.

Example 2.

To test the stability of the not selected, PEG-treated population one of the above cellines (PEG 4) was further subcultured without any selective pressure for a period of 2 month and was then re-evaluated for factor VIII activity. These cells still expressed a higher amount of rVIII SQ.

Table 2. Stability of increased expression capability of PEG treated cells. VIII:C expression compared to control after 60 days in culture.

	Day 61	Day 62	Day 63	Day 64	Day 65	Day 66	Day 67	Day 68
Control	100%	100%	100%	100%	100%	nt.	nt	100%
PEG 4	100%	907%	1892 %	1541 %	1187 %	nt	nt	294%

Result

The expression from day 62 to 68 after fusion, kept under nonselective conditions was between 294 % and 1892 % of the control values.

Example 3

The already PEG fused celline (PEG 4) was again PEG fused with itself, and further subcultured for 10 days without any selective pressure.

Table 3. VIII:C expression after retreating PEG treated cells (PEG 4) with PEG compared to non treated control cells.

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Control	100%	100%	100%	100%	100%	100%	100%
PEGx2	100%	1933%	1884%	1799%	nt	nt	490%

Result

The twice "fusion treated cells" expressed even higher levels of r-VIII SQ compared to the single fused cells. Thus PEG treatment can be performed repeatedly.

Example 4

The celline PFT was further examined by chromosome counting and DNA content by fluorescence after propidium iodide staining. Table 4 shows the results of chromosome analysis after 10-20 days in culture after fusion on two different occasions. At these timepoints an increased expression as described above was present. All cellines show a modal chromosome number of 20. All examined cells showed the presence of aberrant metaphase plates with subtetraploid chromosome numbers. There was however no differences comparing the fused cells to the control celline. This was further studied by propidiumidodine staining and analysis of DNA content fluorescence analysis on several occasions during the propagation of the cells.

Table 4. Chromosome analysis. No of metaphases with the indicated chromosome number in the PEG treated cells and in the control

Chrom numb	18	19	20	21	22	24	32	34	36	37	38	39
Control		6	8	4						1	1	
ADLA PFT 1	2	2	7	3	1			2	1			1
ADLA PFT 2	1	3	11	2		1	1	1				

Result

The PEG treated cells did not have a higher DNA content compared to the non fused control cellines. These analysis, together with the fact that the high expression levels were stable after long time non selective cell culture indicate that the high producer cells were not the result of a classical cellfusion process.

Discussion

Cellfusion is a well-known process. The examples presented here are though not typical for classical cellfusion. Firstly the PEG treated cells contained neither more DNA nor chromosomes and secondly there were no selective steps involved in subculturing the population. Furthermore the regular fusion frequencies of 10^{-4}-10^{-5} are to low for cellfusion as explanation of the long term persistence of the increased productivity. Taken together these results indicate that cellfusion with PEG treatment induce a stable change in the expression capability of the cells. As such this method is applicable to different cells and different agents.

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CLAIMS:

1. A method of obtaining a higher product expression in a producing cell line comprising fusing cells of the same origin with a fusion agent to form fused cells which produce a product.

2. The method according tc claim 1, wherein the fusion agent is polyethylene glycol (PEG).

3. The method according to claim 1 wherein fusion is achieved by the use of electrofusion, protoplasts or Sendaivirus.

4. The method according to any one of claims 1 to 3, in which the product is a therapeutically active protein.

5. The method according to any one of claims 1 to 4, in which the product is full length factor VIII, or a modified factor VIII.

6. The method according to claim 5 wherein the modified factor VIII is a deletion derivative of factor VIII.

7. The method according to claim 6 wherein the deletion derivative of factor VIII is rVIII SQ.

8. The method according to any one of claims 1 to 7, in which the fusing of the cells is performed without the presence of a selection protocol.

9. The method according to any one of claims 1 to 8, in which a selection protocol is used.

10. The method according to any one of claims 1 to 9, in which the fusing of the cells is performed on already fused cells.

11. The method according to any one of claims 1 to 10, in which the cells are Chinese Hamster Ovarian (CHO) cells or are from a Chinese Hamster Ovarian (CHO) derived cell line.

12. The method according to any one of claims 1 to 10, in which the cells are mammalian cells.

13. The method according to any one of claims 1 to 10 wherein the cells are selected from the group consisting of Baby Hamster Kidney (BHK) cells, cells derived from BHK cell lines, Cos cells and cells derived from Cos cell lines.

14. Use of fused cells formed by the method defined in any one of claims 1 to 13, for producing a protein.

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