Survival of 3T3 cells expressing or co-expressing bFGF and/or IGF-I and/or IGF-II in low serum and serum free media

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Abstract

The aim of this study was to improve the survival of NIH3T3 cells in low serum or serum-free media by the endogenous expression of recombinant bFGF (basic fibroblast growth factor) and/or IGF (insulin-like growth factor) -I and II. Expression was detected by Western blotting, and the growth characteristics of different transfected cell lines investigated in both serum-free and low serum conditions and also in soft agar. Morphological changes and the growth rate were compared with growth in normal serum-containing medium. The experimental data suggested that the expression of either bFGF alone, or the co-expression of bFGF and either IGF-I or II could improve the survival of NIH3T3 cells in low serum or serum-free media. The use of such lines could decrease the use of serum in cell culture and thus both reduce the costs involved in this technique and simplify the down-stream purification procedure in protein harvest. Hence, such lines may be of value in both experimental and industrial applications.

Abbreviations: IGF, insulin-like growth factor; bFGF, basic fibroblast growth factor

Introduction

Eukaryotic cells, in contrast to many micro-organisms, are incapable of synthesizing a large number of compounds necessary for their normal functions. These obligatory substances have to be supplied in the growth medium, often as a serum supplement. Since the 1940s, more and more of the components of serum that are important for cell culture have been identified and this has provided the foundation for more systematic media developments. Even with these efforts, however, many cells still require a small amount of serum or serum substitutes to be added for continued growth.

Serum is a complex mixture containing a large number of constituents, and the exact nature of some of these constituents remains unknown. Thus, the addition of serum introduces an element of uncertainty into the art of cell cultivation. Moreover, there is particular concern about the biological complexity

and variability inherent in serum. Additionally, the isolation or purification of a particular product from cell culture is complicated by the presence of serum, which can both clog filters and complicate chromatograms. Finally, contamination of pharmacological products with minute amounts of serum components may seriously affect their quality. Indeed, the international regulations for good manufacturing practice (GMP) for vaccine production restrict the amount of serum constituents in the final product when making injectable vaccines, since allergic reactions could be provoked (Bjare, 1992). It is thus obvious that the replacement of serum by a substitute of defined composition would remove several significant problems associated with cell culture (e.g. precise specification of culture conditions) and would also simplify the down-stream purification procedure in protein harvest.

Two possible strategies exist to decrease the use of serum in culture media. One is to make up a 'defined medium' in which the constituents of the medium

(a) NIH3T3

(b) 3B

Figure 1a-b. Cells of NIH3T3 (a) and 3B (b) in DMEM, with 10% serum, 5% serum and 0% serum (from left to right) after cultivation for 24 hr (x200).

mimic the components of the serum. As more and more constituents in serum are identified, the 'defined medium' becomes more and more authentic. Another approach is to introduce the genes for certain growth promoting factor(s) into a cell line and use genetic engineering techniques to express them in the cell, so that an autocrine loop can be formed.

It has been observed that basic fibroblast growth factor (bFGF) stimulates the sustained proliferation

of mouse epidermal melanoblasts derived from epidermal cell suspensions and neural cells in a serum-free medium supplemented with dibutyl adenosine 3', 5'-cyclic monophosphate (dbcAMP) (Hirobe, 1992; Kitchens et al., 1994). Halaban et al. (1987) cultured human epidermal melanocytes in a serum-free medium supplemented with basic FGF and dbcAMP or basic FGF and 12-O-tetradecanoyl-13-acetate (TPA), respectively. Gordon et al. (1989)

(c) 3I

(d) 3BI

Figure 1c-d. Cells of 3I (c) and 3BI (d) in DMEM, with 10% serum, 5% serum and 0% serum (from left to right) after cultivation for 24 hr (x200).

reported that basic FGF replaced the proliferation-stimulating effect of keratinocytes in a co-cultivation system.

Insulin-like growth factor I (IGF-I), often combined with other hormones and growth factors, has been used as a culture medium supplement (Conover et al., 1989). A number of human tumour cell lines and foetal neurone cells have been shown to synthesize insulin-like growth factors (IGFs) and insulin-like

growth factor binding proteins (IGFBPs) and to have IGF-I or insulin-like growth factor II (IGF-II) receptors (Macaulay, 1992; Schechter et al., 1995; Singleton et al., 1996; Yang et al., 1996). Some of these cell lines grow in the absence of exogenous growth factors, and proliferation in serum-free media can be inhibited by antibodies that bind IGF-I, IGF-II, or the IGF-I receptor (Cullen et al., 1992). Thus the IGFs may serve as autocrine regulators of tumour growth in these cells.

(e) 3II

(f) 3BII

Figure 1e-f. Cells of 3II (e) and 3BII (f) in DMEM, with 10% serum, 5% serum and 0% serum (from left to right) after cultivation for 24 hr (x200).

In this study DNA transfection was used to introduce expression constructs containing the genes for basic FGF, IGF-I and IGF-II into mouse fibroblast NIH3T3 cells. The effect of the expression of these genes on the host cells when cultivated in low-serum and serum-free medium was investigated. Basic FGF cDNA under the control of the human cyto-

megalovirus immediate early (CMV) promoter was inserted into a retroviral vector, pLNCX, so that the resulting plasmid (pDJSF4) could be introduced into cells by either plasmid DNA transfection or retrovirus infection. The cDNAs of IGF-I and IGF-II were inserted separately into another eukaryotic expression vector, pCMVhyg, in which they too are expressed

Figure 2. Western blotting for bFGF with standard (lane 1), 3B (lane 2), 3BI (lane 3), 3BII (lane 4), and 3LN (lane 5) and NIH3T3 (lane 6) as negative controls. The bands in lanes 2–4 are seen to co-migrate with the band in the standard lane, which has a size of 14.5 kDa.

from the CMV promoter. Since these two different vectors (pLNCX and pCMVhyg) drive the inserted genes with the same promoter, levels of expression of basic FGF, IGF-I and IGF-II should be similar. The use of different selectable markers (*neo^r* – pLNCX; *hyg^r* – pCMVhyg) means that basic FGF and IGF-I or basic FGF and IGF-II could be introduced into the same cell line by sequential calcium phosphate precipitation. Expression of basic FGF, IGF-I and IGF-II was confirmed by immuno-blotting and the behaviour of these different cell lines in low serum or serum-free media was investigated.

Materials and methods

Plasmids

pLNCX is a non-splicing retrovirus vector in which the exogenous gene and antibiotic selectable marker are expressed from different promoters. The former gene is driven by the CMV immediate early promoter, and the latter by the MoMLV LTR promoter (Miller and Rosman, 1989). pCMVhyg is a high level mammalian expression vector which contains the gene for hygromycin resistance. The exogenous inserted gene is driven by the human cytomegalovirus immediate early gene enhancer and promoter.

Plasmid pDJSF1, which contains bovine bFGF cDNA lacking the 3' non-coding sequence, has been described (Li, 1997).

Table 1. Growth factor expression plasmids

Plasmid	Designed to express
pDJSF4	bFGF
pDJIGF-I	hIGF-I
pDJIGF-II	hIGF-II

Cell line

The NIH3T3 cell line (ECACC, Porton Down) is highly contact-inhibited and has morphological characteristics which are well suited to transformation assays.

Culture media

All media components were purchased from Life Technologies, (Paisley).

The cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) with 10% FBS (foetal bovine serum), 7.5% FBS, 5% FBS, 2.5% FBS, or without FBS, and containing penicillin (50 IU/ml) and streptomycin (50 µg/ml).

Soft agar media, was made from DMEM with 20% FBS, and contained penicillin (100 IU/ml) and streptomycin (100 µg/ml). It consisted of bottom agar (2 × DMEM medium containing 0.66% agar) and top agar (2 × DMEM medium containing 0.33% agar).

Construction of expression vectors of bFGF, IGF-I and -II

Three vectors were constructed for the expression of bFGF, IGF-I and IGF-II in NIH3T3 cells for use in this study as follows: plasmid pDJSF4 was constructed by inserting bFGF cDNA obtained from plasmid pDJSF1, into pLNCX. Plasmids pDJIGF-I and pDJIGF-II were constructed by inserting the cDNAs of IGF-I and II, obtained from pHJIGF-I102 and pHJIGF-II200 (derivatives of pBluescript containing human IGF-I or II cDNAs) respectively, into pCMVhyg. The main relevant features of these plasmids are summarised in Table 1.

Transfection

NIH3T3 cells were seeded at 5×10^5 cells/10 cm dish in 10 ml medium and incubated overnight at 37 °C. 0.25 M CaCl₂ (0.5 ml) was placed in a sterile 15 ml

Figure 3. Western blotting for IGF-I in 3I and 3BI media. Lane 1: purified IGF-I (standard). Lane 2: serum free medium. Lane 3: medium from 3T3 cell cultures. Lanes 4–6: medium from 3I cell cultures. (lane 4 – after 5 hr culture; lane 5 – after 10 hr culture; lane 6 – after 15 hr culture). Lanes 7–9: medium from 3BI cell cultures (lane 7 – after 5 hr culture; lane 8 – after 10 hr culture; lane 9 – after 15 hr culture). Lane 10: purified IGF-I kept in serum free medium at 37 °C for 5 hr. IGF-I is expressed in both the 3I and 3BI cell lines, but is denatured in serum-free medium. This is shown in lane 10, which contains a sample of serum-free medium (DMEM) in which pure IGF-I (5 ng) had been incubated for 5 hr at 37 °C. The band of the size expected for IGF-I (7.5 kDa) disappeared and novel high molecular weight bands (80–85 kDa) appeared.

Falcon tube, and plasmid DNA (1 mg/ml in TE) added. HEPES-buffered saline (0.5 ml of 2x) was added dropwise with constant swirling and the suspension left at room temperature for 20 min before addition dropwise to the cells. After incubation for 4–6 hr at 37 °C the medium was aspirated and the cells rinsed with 10 ml of PBS. Glycerol (2 ml of 15%) was added to the dish with swirling for 1 min. The glycerol was aspirated, the cells rinsed twice with 10 ml of PBS, twice with 10 ml of growth medium and then fed with fresh growth medium. After incubation at 37 °C for 24 hr the cells were trypsinized and split at an appropriate ratio (>1:10). After a further 24 hr growth clones were selected which were expressing the transfected sequences.

Selection for transfectants

Cells were incubated for 9 days (with media changes every 3 days) in media containing geneticin at 500 µg/ml or hygromycin at 200 µg/ml as appropriate. Dishes were screened by inverted phase microscopy and clones were picked into 24 well culture plates for recovery.

Five cell lines expressing the growth factors either singly or in combination were established for the investigation of cell survival in serum-free medium; the

Table 2. Cell lines generated

Name	Precursor cell	cDNA(s) transfected
3B	NIH 3T3	bFGF
3I	NIH 3T3	hIGF-I
3II	NIH 3T3	hIGF-II
3BI	3B	bFGF, hIGF-I
3BII	3B	bFGF, hIGF-II

main relevant features of these are summarised in Table 2.

Assay for detection of bFGF, IGF-I and -II expression (Western blotting)

Transfected and non-transfected NIH3T3 cells (1×10^8) were lysed in 1 ml of lysis buffer containing 50 mM TrisHCl buffer (pH 7.5), 400 mM NaCl, 1 mM MgCl₂, 1% Nonidet P-40 and 1 µM phenylmethylsulfonyl fluoride. Nuclei and cell debris were removed by centrifugation at $16000 \times g$ for 10 min at 4 °C. The supernatants were analysed for bFGF, IGF-I and IGF-II by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) and Western blotting on nitrocellulose. The Western blots were probed with affinity-purified mouse anti-bFGF (Sigma), IGF-I and

Figure 4. Western blotting for IGF-II in samples of 3BII media. Lane 1: purified IGF-II. Lane 2: serum free medium. Lane 3: medium taken from 3T3 cell cultures after 12 hr. Lane 4: medium taken from 3T3 cell cultures after 24 hr. Lanes 5-9: medium from 3BII cell cultures (lane 5 – after 5 hr culture; lane 6 – after 10 hr culture; lane 7 – after 15 hr culture; lane 8 – after 15 hr culture; lane 9 – after 25 hr culture). Lane 10: purified IGF-II kept in serum free medium at 37 °C for 5 hr. IGF-II is expressed in the cell line 3BII but is denatured in serum-free media. This is shown in lane 10, which contains a sample of serum-free medium (DMEM) in which pure IGF-II (5 ng) had been incubated for 5 hr at 37 °C. The band of the size expected for IGF-II (6 kDa) disappeared and novel high molecular weight bands (80–85 kDa) appeared.

IGF-II antibodies (Serotec Inc.). The second antibody used for luminescence development was horseradish peroxidase-conjugate rabbit Ig against mouse Ig as described by the manufacturer (Amersham Lifescience, ECL system).

Results and discussion

Cell line 3B

NIH3T3 cells were cultured until 70% confluent and transfected with 20 μ g Qiagen-grade DNA/10cm dish of pDJSF4 and pLNCX, respectively. No changes were observed in pLNCX-transfected cells (Figure 1a), whereas foci isolated following transfection with pDJSF4, e.g. the cell line 3B, grew as non-adherent clusters of large refractive cells (Figure 1b).

At mitosis, cells were more refractive, rounder in shape and had longer, thinner processes. At confluence, 3B cells were reminiscent of NIH3T3 cells, but had a less organised morphology.

Cell lines 3I and 3BI

The cell lines NIH3T3 and 3B were transfected with plasmid pDJIGF-I to produce lines 3I and 3BI, respectively (Figures 1c and 1d). Their morphology was more transformed than that of NIH3T3 cells, and that

was particularly true of all the foci originating from the 3B cells.

The 3I cells had an exaggerated bipolar form, small body size, spindle-like shape and shorter cytoplasmic processes than 3B cells. 3BI cells resembled 3I cells, but were more frequently seen in mitosis.

Cell lines 3II and 3BII

3II and 3BII (Figures 1e and 1f) were isolated from NIH3T3 and 3B cells respectively, following transfection with plasmid pDJIGF-II.

3II cells resembled the 3I cell line, but a smaller percentage of 3II cells were in mitosis, and flattened, non-mitotic cells dominated the cultures. A high proportion of 3BII cells had lost the morphology typical of 3B cells and reverted to an appearance which was more like that of NIH3T3 cells. They could be easily distinguished from 3B cells by their firm attachment to the surface of the flask, their flattened cell shape, and their cell body size, which was as small as NIH3T3 cells. The 3BII cell culture contained far fewer mitotic cells than the 3B cell culture. At confluence, the cells remained as a monolayer and assumed an organised sheet without any irregular criss-cross pattern. These morphological changes suggested that the changes which arose as a result of bFGF expression in the 3B cell could be counteracted by the co-expression of IGF-II.

Figure 5. Number of viable cells of each line after cultivation for 6 days in media containing different percentages of serum.

Figure 6. Numbers of cell colonies formed after 10 days growth from 1000 cells seeded into soft agar medium in a 6 cm Petri dish.

bFGF expression in cell lines 3B, 3BI and 3BII

Western blotting to detect bFGF was carried out on fresh cell lysates and serum-free culture media from cell lines 3B, 3BI, 3BII, 3LN (NIH3T3 cells transfected with pLNCX plasmid DNA) and NIH3T3. A distinct band at 14.5 kDa, coincident with that in the bFGF control, was detected in the cell lysate samples of cell lines 3B, 3BI and 3BII, with only a faint band being observed in the lanes of samples derived from 3LN and NIH3T3 cells. It was, therefore, concluded that the transfected bFGF cDNA had been expressed in the 3B, 3BI and 3BII cells and that transfection with IGF-I or IGF-II did not interfere with bFGF expression (Figure 2).

IGF-I expression in cell lines 3I and 3BI

Both cell lysates and conditioned serum-free medium from cultures of 3I and 3BI were assayed for the presence of IGF-I. IGF-I was detected by Western blotting

in the medium prepared from 3I and 3BI cells, but not in any cell lysates (Figure 3). However, two bands were observed in the culture media from 3I and 3BI whereas only one band was present in the positive control lane. The content of IGF-I in the medium continuously increased as the duration time of incubation increased.

IGF-II expression in cell lines 3II and 3BII

Expression of IGF-II was investigated in both cell lysates and conditioned serum-free medium from cultures of 3II, 3BII and NIH3T3 cells. IGF-II could not be detected in any cell lysates but was detectable by Western blotting of conditioned medium from 3BII cells (Figure 4), but not 3II cells (not shown). The level of IGF-II in the medium increased with increased duration of incubation and it was noted that IGF-II is denatured in serum-free media.

Growth of cell lines in low serum and serum-free media

Cells of the NIH3T3, 3B, 3BI, 3BII, 3I and 3II lines were seeded at a density of $4 \times 10^5/25 \text{ cm}^2$ flask in three flasks for each of five serum concentrations (0, 2.5, 5, 7.5 and 10%). They were incubated for six days, with medium replacement after three days, examined and photographed daily, and on the sixth day, they were harvested and counted. Figure 5 shows the number of viable cells present in each of these cultures after six days cultivation. In low serum medium, the cells of 3B, 3I, 3II, 3BI and 3BII grew better than NIH3T3 cells, with 3B, 3I and 3BI cells showing the most marked differences.

Morphological changes in low serum and serum-free media

Figure 1 shows the transfected cell lines, 3B, 3BI, 3BII, 3I, 3II and the negative control, NIH3T3, after cultivation for 24 hr in 10, 5 and 0% serum at a seeding density of $4 \times 10^5/25 \text{ cm}^2$ flask. The morphological changes were as follows:

NIH3T3: at a serum concentration of 5% vacuoles appeared in the cytoplasm of some cells and the cellular processes became twisted. In the complete absence of serum there was much cell debris, numerous vacuoles could be found inside the viable cells, and the number of cells present decreased drastically (Figure 1a).

3B: there were no visible changes in the morphological appearance of 3B cells at a serum concentration of 5%. However, in serum-free medium, tiny vacuoles were found inside the viable cells and the cells looked less healthy, although no dead cells were present and the number of cells did not decrease compared with the original number seeded (Figure 1b).

3I and 3BI: the morphological appearance of these cell lines was similar and they showed little change in appearance in 5 and 0% serum compared with 10% serum. They were spindle-like in shape and their body size was smaller than 3B. They were still mitotically active and looked very healthy in serum-free media. However, the ability of these cells to adhere to the bottom of the culture flask was not as marked as 3B (Figures 1c and d).

3II and 3BII: the appearance of these two cell lines differed and their growth rate was slower than 3B, 3BI and 3I in low serum or serum-free media. In 10% serum, 3BII cells showed the strongest adhesion to the culture flask of all of the transfected cell lines

and these cells remained in monolayer (showing contact inhibition) after they reached confluence. Their cytoplasmic processes started to twist in 5% serum, most of the processes were lost in serum-free medium and numerous vacuoles could be found inside the viable 3BII cells (Figures 1e and 1f), behaviour reminiscent of that of NIH3T3 cells.

Growth of cell lines 3B, 3I, 3II, 3BI, 3BII and NIH3T3 in soft agar media

Cells were seeded into soft agar at a density of 1000 cells/6 cm dish and after 10 days incubation were observed under the inverted microscope. Cell colonies were visible in all the lines except NIH3T3. The ability of these lines to form colonies in soft agar was as follows: 3BI > 3BII > 3B > 3I > 3II (Figure 6).

The aim of the study was to test the effect of the expression of bFGF and/or IGFs in NIH3T3 cells on their ability to survive in low serum or serum-free media. All of the cell lines, NIH3T3, 3B, 3BI, 3BII, 3I, and 3II, showed altered growth behaviour at decreased serum concentrations over a 6-day cultivation period, with differences apparent between the cell lines. The ability of 3B, 3BI and 3I cells to grow in low serum and serum-free conditions was greater than that of 3II and 3BII cells. In general terms, 3B gave the best growth in low serum and serum-free media conditions. For example, in 2.5% serum, this cell line reached the highest final viable cell count, a density of 2.1×10^6 cells/ml. In serum-free medium, the viable cell numbers for 3B, 3BI and 3I were twice as high as NIH3T3 after 6 days cultivation. However, the lines 3II and 3BII showed a lower ability to survive than the cell lines mentioned above. After 6 days cultivation in serum-free media, the viable cell number for 3BII and 3II was less than half the initial seeding number but was similar to the NIH3T3 cells under the same conditions.

The cell line 3I showed a higher growth rate than the cell line 3II, and the growth rate of the cell line 3BII was much lower than that of the cell lines 3B and 3BI. The 3BII cells remained in monolayer (showing contact inhibition) for 5 days after they reached confluence, whereas 3B and 3BI cells overlapped each other immediately after they reached confluence. The body size of 3BII cells was greater than the other transfected cell lines. These characteristics suggested that the 3BII cells were morphologically less transformed. However, 3BII cells produced more clones than 3B in soft agar medium. These conflicting results raise a

question as to which of the well-established criteria for assessing the degree of cellular transformation (loss of contact inhibition, ability to grow in soft agar, increase in cell growth rate or ability to survive in serum-free medium) is the most reliable.

The results from this study have demonstrated that the endogenous expression of IGF-I and IGF-II enhanced the growth of NIH3T3 cells and improved their ability to survive in low serum and serum-free media.

There have been no reports published describing the expression of bFGF and IGF-I or bFGF and IGF-II in the same cell line and the effects of such expression on the growth of the cells. IGF-I and -II have been reported to have similar biological functions even in different cell types, although IGF-I exerts a stronger effect on cells than IGF-II (Froger-Gaillard et al., 1989; Taylor et al., 1988; Kemp et al., 1988). IGF-I plays the main role in growth and metabolic promotion in adult mammalian cells, and IGF-II in foetal tissues (Vetter et al., 1986). However, in this study, differences were observed in the growth of cells transfected with bFGF/IGF-I or bFGF/IGF-II, and these could be considered to be opposite rather than 'strong' or 'weak' effects. Thus, we conclude that the co-expression of bFGF/IGF-I or bFGF/IGF-II have different effects on the growth of transfected cell lines.

Conclusions

Co-expression of bFGF and IGF-I or -II in NIH3T3 cells can improve the growth of cells in low serum or serum-free media in terms of morphological changes and their viability compared with NIH3T3 cells. Use of lines expressing these growth factors could therefore result in a decrease in the use of serum and a simplification of downstream purification procedures. Thus, this work has implications for the manufacture of biological products in industry using genetically engineered animal cells.

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