Manipulation of culture conditions for BHK cell growth inhibition by IRF-1 activation

Ana V. Carvalhal¹, José L. Moreira¹, Helder Cruz¹, P. Mueller², Hansjörg Hauser² & Manuel J.T. Carrondo^{1,*}

¹ Instituto de Biologia Experimental e Tecnológica/Instituto de Tecnologia Química e Biológica IBET/ITQB, Apartado 12, 2780 Oeiras, Portugal

² GBF-Gesellschaft für Biotechnologische Forschung mbH., Mascheroder Weg 1, 38124 Braunschweig, Germany

³ Laboratório de Engenharia Bioquímica, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, P-2825 Monte da Caparica, Portugal

(* Author for correspondence)

Received 5 January 1999; accepted 18 October 1999

Key words: BHK, cell growth inhibition, estradiol, IRF-1, reversibility

Abstract

The activation of interferon-regulatory-factor-1 (IRF-1) has been applied to regulate the cell growth of BHK cells. The constitutively expressed IRF-1-estrogen receptor fusion protein (IRF-1-hER) activated by the addition to the culture medium of an estrogen analogue (estradiol), enabled IRF-1 to gain its transcriptional activator function. By using a dicistronic stabilised self-selecting construct it was possible to control cell proliferation. With the addition of 100 nM of estradiol at the beginning of the exponential phase, the IRF-1 activation led to a rapid cell growth inhibition. Two days after estradiol addition cell concentration was still maintained but a decrease in cell viability was observed. This cell response is independent on clone (producer and non-producer) and culture system (static and stirred cultures). Specific recombinant-protein productivity of the producer clone was not significantly altered. Control experiments confirmed that IRF-1 activation effect was not due to the addition of estradiol *per se*, estradiol solvent or serum concentration. The extent of cell growth inhibition is dependent on estradiol concentration and estradiol addition time, although a decrease in cell viability was always observed. Reducing the time span of estradiol exposure allowed the decrease in the cell viability to be controlled and the stationary inhibited phase to be extended: when the time of contact between the cells and estradiol is reduced cell viability increases, achieving values similar to those obtained if no estradiol is added. During this recovery phase the cells passed two different phases: first a stationary phase extension where cell growth was still inhibited, followed by an increase of cell concentration. The IRF-1 system is reversible. This pattern can be repeated for an extended period when estradiol addition and removal are repeated, showing a cyclic response. Thus, it is possible to modulate the IRF-1 effect by manipulating cycles of addition/removal of estradiol and in this way the stationary phase can be maintained.

Introduction

As a defence against viral or bacterial infection and certain tumours, mammalian cells secrete interferons (IFN) that bind to cell surface receptors to protect the neighbouring cells and prevent propagation of the virus (Pellegrini and Schindler, 1993). Amongst the genes that are induced in response to IFN is the interferon-regulatory-factor-1 (IRF-1), which can it-

self modulate the expression of interferons and of some interferon-inducible proteins (Harada et al., 1989; Kimura et al., 1994).

Interferons inhibit the proliferation of susceptible cell types. In part this may be due to the induction of IRF-1 that may function by activating sets of genes involved in the negative regulation of cell growth (Harada et al., 1994). In fact, IRF-1 shows tumour suppressor activity, the loss of functional IRF-1 being

proposed as the reason for numerous cases of myelodysplasia syndrome and leukaemia (Gothelf et al., 1991; Tanaka et al., 1994; Willman et al., 1993).

In vitro cell growth inhibition was observed when IRF-1 is activated (Kirchhoff et al., 1993; Koromilas et al., 1992).

Regulating the activation of IRF-1 permits not only a better understanding of the role of this protein as an activator of genes (essential for the study of cell growth), but can also be used for other purposes such as cancer therapy or better controlled bioreaction processes.

Growth inhibition was observed for several cell lines (C243, NIH 3T3, BHK-21 and Ltk⁻) after activation of IRF-1-hER (Kirchhoff et al., 1993, 1995, 1996; Köster et al., 1995; Matsuyama et al., 1993). In these studies the regulation of intracellular concentration of the active IRF-1 was achieved through the use of a fusion protein composed by the IRF-1 gene and the human estrogen receptor (hER), which, in turn, has an high affinity for steroid hormones like estradiol, an analogue of estrogen (Kirchhoff et al., 1993, 1995, 1996).

Previous studies have shown that this system is efficient in inhibiting cell growth of a BHK-21 clone (Kirchhoff et al., 1996). This concept can also be applied for bioreaction purposes: it could be expected that when cell proliferation is inhibited cells could devote more of their metabolic activity and energy to the synthesis of the product of interest.

The present work concerns the application of the IRF-1-hER activation concept on bioreaction for the regulation of BHK cell growth. The effect of this concept upon the productivity of Factor VII is also studied. Factor VII, the protein of interest expressed in the clone used in this work, is a 50 Kd single chain glycoprotein involved in the blood coagulation process (Bajaj et al., 1981; Broze and Majerus, 1980). Proliferation dependence upon estradiol concentration, estradiol addition time and time span of estradiol exposure is evaluated. In order to prove that the proliferation inhibition is not a result of a secondary effect it was confirmed that both solvent and serum have no influence and that there is no nutrient depletion.

Materials and methods

Cell line and gene transfer

The dicistronic IRF-1-hER expression plasmid has

been constructed as follows: poliovirus internal ribosome entry site (IRES) – puromycin acetyl transferase (PAC) DNA fragment has been isolated by cleavage of PMC-2P with EcoRI and NheI and ligated to the EcoRI-NheI cleaved vector IRF-1-hER (Kirchhoff et al., 1993) carrying the IRF-1-hER gene and ampicillin resistance for propagation in *E. coli* (Figure 1).

BHK-21A cells (ATCC CCL10) and BHK-21A cells expressing blood coagulation Factor VII (FVII) (non-regulated/producer clone, kindly supplied by Dr Leif Kongerslev from Novo Nordisk, Gentofte, Denmark) were stably transfected using the calcium coprecipitation method (Wigler et al., 1977) and the transfectants selected in DMEM with 10% FCS that has previously been tested to support high transfection efficiencies of IRF-1-hER encoding plasmids. Single clones were picked from the petri-dishes and transferred to 96-well plates. Growth at different cell densities was compared after 5 days in the presence or absence of 100 nM estradiol. Single clones that showed growth inhibition in the presence of estradiol were further propagated for six months and tested for estradiol sensitive growth at weekly intervals. After this period, one stable growth regulated/non-producer clone out of 23 puromycin resistant clones and two regulated/producer clones (I and II) out of 16 puromycin resistant clones were chosen.

Culture medium

The IRF-1-hER regulated BHK clones were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS), 4.5 g L⁻¹ of glucose, 5 mg L⁻¹ of puromycin and 5 mg L⁻¹ of vitamin K1 (all final concentrations). Unless indicated, FBS was supplied by Sigma (St. Louis, MO) ref. nr. F-7524. All others were supplied by Life Technologies (Glasgow, UK). The non-regulated/FVII producer clone was grown in a 1:1 mixture of DMEM and Ham's F12, supplemented with 10% (v/v) FBS, 4.5 g L⁻¹ of glucose and 5 mg L⁻¹ of vitamin K1 (all final concentrations), all from Life Technologies except the serum.

Serum from other brands than Sigma were also used: Life Technologies (normal serum ref. nr. 10270–16 and dialysed serum ref. nr. 10110–161) and Hyclone (Utah, U.S.A.) (normal serum ref. nr. SH30070 and charcoal/dextran treated serum ref. nr. SH30068).

The cells were grown in a humidified atmosphere of 7% CO₂ at 37 °C.

Figure 1. Construction of a dicistronic transcription unit for the stabilization of recombinant gene expression. The expression of the growth regulatory reading frame (IRF-1-hER) was transcriptionally linked to the expression of an antibiotic drug selection marker (PAC) using an internal ribosomal entry site (IRES) that allows the 5' cap independent translation initiation at downstream AUG codons. Both reading frames are transcribed from a single constitutive promoter (P) and the dicistronic transcript is terminated at a downstream poly A addition site (A_n).

Culture system

25 cm² static T-flasks (Greiner, Nurtingen, Germany) were used for static culture studies. Seeding density of 2×10^4 cells cm⁻² was used for a total medium volume of 10 mL. Bioreaction studies were performed in 250 cm³ (working volume) spinner-flasks (Wheaton, Techne, U.S.A.) at an agitation rate of 80 rpm, with the cells immobilised as natural aggregates. A concentration of 2.5×10^5 cells mL⁻¹ was used to inoculate each spinner flask.

Sampling

Evaluation of cell concentration as well as cell viability (both adherent and single cell suspension) were carried out by counting cells on a haemocytometer using the trypan blue dye exclusion test. Aggregate characterisation in spinner flasks was performed as described by Moreira et al. (1994).

Glucose and lactate measurements were performed by GPLC and Factor VII concentration quantified by a normal ELISA assay, with the specific antibodies being supplied by Dr Leif Kongerslev from Novo Nordisk.

Estradiol preparation

17 β -Estradiol (MERCK, Darmstadt, Germany) dissolved in ethanol (final concentration 1 mM), was added to the culture medium with a dilution of 1:10000, unless otherwise indicated, for a final concentration of 100 nM. Its removal from the culture medium was performed by washing the cells with medium and then replacing the total supernatant by fresh medium. Ethanol was present in the culture medium at a concentration of 0.01% (v/v), and no toxic effects were observed, in agreement with Kasajima and Tsutsui (1990) and Ahluwalia (1995).

Proliferation assay

For each test two experiments were always done in parallel using cells from the same origin and prepared exactly in the same way. In one experiment estradiol was added as described below and in the other it was used as an internal control (without estradiol, therefore without IRF-1 activation). These experiments were repeated at least two times, the same pattern being observed.

Results and discussion

Cell growth and viability in batch growth controlled cultures

Aiming at a stronger stabilisation of IRF-1-hER expression, the selection marker PAC has been transcriptionally linked in a dicistronic construct separated from IRF-1-hER by the IRES to allow translation of the second reading frame. This represents a different construct from the monocistronic system studied by Kirchhoff et al. (1995), where the cells were co-transfected with two separate plasmid DNAs encoding IRF-1-hER and PAC. Proliferation controlled single clones were propagated in the presence of puromycin and the growth inhibitory response to estradiol examined, showing that the clones transfected with the dicistronic construct presented a greater stability maintaining the proliferation control system after 40 passages.

Factor VII producer cells transfected with this dicistronic construct were tested. The preliminary tests performed with the regulated/FVII producer clone I in static batch cultures aimed to detect changes in the growth pattern resulting from the activation of the constitutively expressed IRF-1 fusion protein after the

Figure 2. Effect of estradiol addition in static culture (added at the beginning of the exponential phase, 48 hours after inoculation, 100 nM estradiol) upon cell concentration (A) and viability (B) (regulated/FVII producer clone). □ Control; ▲ Addition to a final concentration of 100 nM estradiol to the culture medium (at +E).

addition of estradiol. In order to eliminate the interference of external factors such as nutrient depletion, product toxicity or cell growth surface limitation, 100 nM of estradiol was added at the beginning of the exponential growth phase (this estradiol concentration proved to be efficient for cell growth inhibition during short periods of culture, data not shown). As previously described in Materials and methods, an internal control using similar cells without the addition of estradiol was performed in parallel. The results are presented in Figure 2.

Cell growth is clearly reduced in the presence of estradiol; 48 hours after estradiol addition the cell concentration in the control increased up to 7×10^5 cells mL⁻¹ (as expected in a normal batch static culture) whereas in the experiment where estradiol was added the cell concentration remained close to 2×10^5 cells

Figure 3. Morphology of adherent cells (static culture): (I) Control (II) 48 hours after estradiol addition.

mL⁻¹ (Figure 2A) (specific growth rate decreased from 0.026 ± 0.001 h⁻¹ to approximately zero). However, a sharp decrease in cell viability occurred at that time in the experiment where estradiol was added (95 versus 68% 48 hours after estradiol addition) (Figure 2B). This difference was even larger at the end of the experiments, a higher cell death rate occurring where estradiol was added (Table 1). Similar results have been obtained for the other producer clone (II) (data not shown). A significant change in cell morphology was observed 48 hours after estradiol addition, when the cell viability decrease started to be evident (Figure 3). The outer membrane became highly irregular and accumulation of intracellular vesicles was observed (Figure 3). Similar experiments were previously reported by Kirchhoff et al. (1996) for shorter periods (72 hours); cell growth inhibition has been achieved, in agreement with Figure 2A, viability differences being negligible, which can be due to the short time of the experiments when compared with those presented in

Table 1. Effect of IRF-1 activation upon cell growth and total cell viability in static culture

	Estradiol addition time	Estradiol concentration	Serum concentration	Maximum total cell concentration (10^6 cells mL^{-1})	Rate of viability decrease ^a (% day^{-1})
	(h)	(nM)	(%)		
Regulated/ Non-producer clone	no addition (control)	–	10%	1.7 ± 0.3	-3.9 ± 0.8
	48 h	100 nM	10%	0.5 ± 0.1	-14.0 ± 0.4
	48 h	100 nM	20%	0.8 ± 0.1	-11.6 ± 0.7
	48 h	10 nM	10%	1.3 ± 0.3	-15.7 ± 2.1
	96 h	100 nM	10%	1.7 ± 0.2	-13.3 ± 0.8
Regulated/ FVII producer clone	no addition (control)	–	10%	1.1 ± 0.1	-3.9 ± 1.5
	48 h	100 nM	10%	0.3 ± 0.1	-21.9 ± 2.3
Non-Regulated clone	no addition (control)	–	10%	1.0 ± 0.2	-8.6 ± 1.3
	48 h	100 nM	10%	0.9 ± 0.2	-7.1 ± 1.1

^a Rate of cell viability measured from 48 hours after estradiol addition up to the end of the experiment.

Figure 2B. Other clones cotransfected with a monocistronic IRF-1 system were studied by the authors under prolonged time cultivation: similar patterns in terms of cell growth inhibition and viability decrease were observed (data not shown).

The cell productivity of the clone with and without cell growth inhibition is also a fundamental question for the application of this concept in bioreaction. Specific productivities, computed for the same time frame during the batch cultivations, led to the conclusion that Factor VII productivity is almost unchanged by estradiol presence (117 ± 20 IU/ 10^6 cell-h and 150 ± 13 IU/ 10^6 cell-h, without and with estradiol, respectively) seemingly independent upon cell growth inhibition. A similar pattern has been obtained for the other FVII producer clone (II) studied (data not shown). Thus, if high cell viability can be maintained, this genetic concept will be of utmost importance for the consistent production of high quality products over long periods, with less cell turnover and less opportunity for genetic shift. When compared with productivity values known for the initial non-regulated/FVII producer clone, the cell productivities reported above dropped approximately two fold. A similar pattern has been previously reported for an IgG producer BHK clone genetically modified with the same IRF-1-hER (Kirchhoff et al., 1996). It is not clear why cell productivity decreased after IRF-1-hER transfection. However, this does not constitute a limitation for the application of

this concept in production since the productivity can be enhanced by using an IRF-1 inducible promoter to express the gene of the product of interest.

In order to clarify if the decrease in cell viability is associated with the production of FVII, similar experiments to those previously presented in Figure 2 have been performed with a regulated/non-producer clone. The results are presented in Figure 4 and Table 1. As can be seen, a similar pattern to that previously described for the producer clones was observed, with a clear cell growth inhibition associated with the addition of estradiol and a significant drop in cell viability a few days after estradiol addition, earlier than in the control. Similar morphological changes to those presented in Figure 3 were also observed (data not shown). It is interesting to observe that both the cell growth inhibition and the cell death kinetics are not as significantly altered as those obtained for the producer clones. Nevertheless, the issue of viability decrease remained, independently of the clone or the expression of the product of interest (similar patterns have been observed for other clones expressing an IgG, data not shown).

Effect of culture additives upon cell behaviour

Several strategies were tried to overcome the cell viability drop after the estradiol addition. The hypothesis of having a chemical interference introduced into the

Figure 4. Effect of estradiol addition in static culture upon cell concentration (A) and viability (B) (regulated/non-producer clone). Control, □; addition at the beginning of exponential phase (+E at 48 hours) to a final estradiol concentration in the culture medium of 100 nM, ▲ and 10 nM, ●; addition at the end of the exponential phase (+E' at 96 hours) to a final estradiol concentration of 100 nM, ◆.

system with the addition of estradiol was also tested; studies were performed concerning the type of serum, estradiol concentration and the solvent used in the estradiol stock solution.

Serum is normally an essential component for survival and growth *in vitro*, and among its major components are the hormones, including the steroid hormones family, to which estradiol belongs.

In this work the cells were grown in medium containing 10% foetal bovine serum, already containing some residual estradiol at the beginning of the cultivation which, in principle, can activate the expression of the IRF-1-hER protein fusion and lead to some cell growth inhibition. However, this was not the case since

the specific growth rate and maximum cell concentration of the IRF-1 regulated clone in the absence of estradiol are, respectively, $0.025 \pm 0.003 \text{ h}^{-1}$ and $1.7 \times 10^6 \text{ cells mL}^{-1}$, normal values for batch static cultures.

In principle, serum can also contain some substances that may induce or contribute to events that lead, after estradiol addition, to the rapid decrease observed in cell viability. To confirm this observation, foetal bovine serum from several different brands, origins and treatments were tested (as described in Materials and methods); from those, particular attention was given to the dialysed serum from Life Technologies and charcoal/dextran treated serum from Hyclone, since both contain reduced amounts of estradiol (personal communication, from the suppliers). The tests were performed with 10% of serum without the addition of further estradiol and the results compared. Cell growth rates and maximum cell concentrations close to those previously referred were obtained in all cases, clearly indicating that the amount of estradiol present in the tested sera is not significant enough to influence the results described above.

The serum concentration in the culture medium was increased up to 20% in similar experiments, and the results obtained show a similar pattern to that previously described (Table 1). Again, these results indicate that serum is not influencing the results obtained after estradiol addition, neither by the residual estradiol present in the serum nor by the presence of substances that could induce cell death after estradiol addition. Thus, IRF-1 activation effect is serum independent, being exclusively controlled by the addition of estradiol to the culture medium.

Estradiol, like other steroids, is hydrophobic, entering into the cell by diffusion through the plasma membrane, and has a life span of hours in the blood plasma; nevertheless, it is a small molecule (MW 272.4), and the estradiol diffusion process is still rapid, the intracellular estradiol concentration being directly related to the estradiol concentration added to the medium without the need to considered the intracellular diffusion of estradiol as a limiting step. Estradiol concentration in the medium was varied within the range from 3 to 1000 nM (final concentration), in similar experiments to those previously described. In all experiments the concentration of solvent in the culture was maintained constant by changing the concentration in the stock solution, in order to separate these two effects. The results obtained with 10 and 100 nM of estradiol are presented in Figure 4 and Table

1. All estradiol concentrations above 100 nM led to similar results to those presented: cell growth inhibition occurs a short time after estradiol addition (Figure 4A), followed by a significant decrease in cell viability (Figure 4B). Rates of cell growth and death were similar regardless of the final estradiol concentration and independent upon cell concentration (data not shown). Below 100 nM estradiol, IRF-1 activation always led to cell growth inhibition, but its extent is significantly dependent upon the estradiol concentration (Figure 4A), cell growth inhibition being delayed when the estradiol concentration decreases. The main difference is not the reduction in specific cell growth (only slightly decreased with the decrease of estradiol concentration), but the onset of cell growth inhibition, which was delayed more than 24 hours when the concentration was decreased to 10 nM; these results were also confirmed in the experiments performed at lower concentrations. In all studies a clear change in cell morphology 2 days after estradiol addition as previously described was observed (Figure 3), regardless of the estradiol concentration.

Nevertheless, the decrease in cell viability is similar to what has been previously observed (Figures 2B and 4B), being faster for 100 nM than for 10 nM estradiol concentration (Figure 4B). This observation, further confirmed at 3 nM estradiol concentration, limits the application of lower estradiol concentrations as a strategy to be used for long culture periods, since a decrease in cell viability is already apparent at the lower estradiol concentration which only barely limit cell growth. This conclusion is in agreement with a previous report where the IRF-1-hER system was also applied (Kirchhoff et al., 1996). Thus, in the following experiments a final estradiol concentration of 100 nM will be used.

The solvent used for preparation of the estradiol stock solution was ethanol. Cell growth of the regulated clone was evaluated with the addition of 0.01% ethanol (without estradiol) to the culture medium. No variation in cell growth, viability or morphology was observed when compared with the control, confirming these low ethanol concentrations do not interfere in the results, in agreement with Kasajima and Tsutsui (1990) and Ahluwalia et al. (1995).

To determine if the estrogen added to the culture medium has any effect on BHK cell growth *per se*, similar experiments to those described (addition of 100 nM estradiol at the beginning of the exponential phase) were performed with the non-regulated clone, i.e., cells that were not transfected with the IRF-1-hER

construct. There was no difference on cell growth, viability or morphology when compared with the control where estradiol was added (Table 1), clearly confirming that the decrease in cell viability is not due to estradiol or solvent solution.

Dependence of cell behaviour upon cell growth phase and cultivation process

In all experiments previously reported, estradiol was added at the beginning of the exponential phase. The possibility of having the observed pattern of cell viability decrease depending upon cell growth phase should also be considered. For this purpose 100 nM estradiol was added at different times post inoculation and thus at different cell growth phases: 72 hours (mid of the exponential growth phase), 96 hours (end of the exponential growth phase) and 120 hours (beginning of the death phase) of the batch cultivation. The results for the addition of estradiol at 48 and 96 hours of culture are presented in Figure 4 and table 1. Cell growth inhibition is clear at the beginning of the exponential phase (Figure 4A). However, at the end of the exponential phase this effect is masked by the natural end of the batch culture, limiting the distinction between the experiment and the correspondent control. A delay in the activation of the IRF-1-hER system by addition of estradiol at later phases of growth is apparent; this can be due to higher cell concentrations, since the amount of estradiol per cell is decreased (in agreement with the results presented in Figure 4). Results obtained by adding estradiol at 72 and 120 hours of culture confirm these observations (data not shown). This is clearly in agreement with the results obtained from the study of estradiol concentration at the seeding inoculum concentration, where it was clear that at low estradiol/cell concentration ratio the rate of cell growth inhibition decreases. Thus, in production conditions the concentration of estradiol in culture to obtain cell growth control should be set based upon cell concentration criteria.

A decrease in cell viability was still observed in all experiments (Figure 4B). Cell death is delayed by the estradiol addition time (Figure 4B), probably not only due to the late addition but also to the lower ratio of estradiol per cell, as previously referred.

From these experiments, it can be concluded that the cell growth phase is neither affecting the cell growth inhibition nor the drop in cell viability; thus, the previously presented results are not specific for the beginning of the growth phase. Nevertheless, the goal

Figure 5. Effect of estradiol addition in stirred culture (added at the beginning of the exponential phase, 24 hours after inoculation, 100 nM estradiol) upon cell concentration (A) and viability (B) (regulated/non-producer clone). □ Control; ▲ Addition to a final concentration of 100 nM estradiol to the culture medium (at +E).

of this part of the work was not to define the best cell growth phase to initiate the inhibition, since for actual operation that would have to be done at the end of the growing phase. However, in order to describe more sharply the estradiol effects, as indicated above, estradiol addition 48 hours after inoculation will continue to be used here.

In order to study the IRF-1-hER system dependency upon the culture method, experiments were carried out for the producer and non-producer regulated clones in small volume stirred spinner vessels operated as batch cultures. The results for the regulated/non-producer clone are presented in Figure 5, confirming the conclusions previously established for static cul-

tures: an effective growth inhibition followed by a decrease in cell viability. This is slightly more pronounced than in static culture (Figure 4) since the cells become more fragile after estradiol addition (IRF-1 activation), this being enhanced by shear stress resulting from the stirred flask agitation. The cell morphology also changed after the IRF-1 activation, with a decrease in the aggregate size and a higher number of single cells in suspension being observed (data not shown). Similar results were obtained for the producer clone in batch stirred flasks.

Effect of time span of estradiol exposure and cycling estradiol addition upon cell behaviour

For all the experiments described above the cells were always in the presence of estradiol during the entire batch cultivation. It is constant in all those results that cell growth inhibition takes place almost immediately after estradiol addition (when enough estradiol is present in the culture medium) and that the cell death only occurs significantly 3 to 4 days after addition. This indicates that a possible strategy to maintain the cell growth inhibited during long periods will be shortening or cycling the exposure of the cells to estradiol.

To test the effect of a shorter cell exposure to estradiol upon cell growth inhibition, estradiol was removed from the culture system by replacing the total supernatant with fresh medium in static culture. As can be seen in Figure 6B, when the time of contact between the cells and estradiol is reduced, the cell viability increases significantly. In Figure 6A it is clear that for a period of 72 hours of estradiol exposure, no significant increase in cell concentration was observed, but when the time of contact was short (24 hours) a slight increase in cell concentration occurred (data not shown). A similar experiment was performed where the estradiol concentration was maintained in the replacement media (data not shown) and cell death was as presented in Figure 4B, confirming that the recovery of viability is due to the removal of estradiol and not to the exchange towards fresh medium. Similar experiments were also performed with the regulated/producer clone, and similar results were obtained (data not shown). Thus, to maintain the cell growth inhibition, it is necessary to have a defined time span of approximately 3 days of estradiol exposure.

The IRF-1 protein is very unstable, with a half-life of 30 minutes (Watanabe et al., 1991; Lengyel et al., 1993). If a somewhat similar instability is assumed for

Figure 6. Effect upon cell concentration in static culture (A) and viability (B) at reduced time of contact between the cells and estradiol (regulated/non-producer clone). □ Control; ○ Addition to a final concentration of 100 nM estradiol to the culture medium at 48 hours (+E); ◇ Addition to a final concentration of 100 nM estradiol to the culture medium at 48 hours (+E), removal of estradiol from the culture medium by replacing the total supernatant by fresh medium at 120 hours (-E); ▲ Addition to a final concentration of 100 nM estradiol to the culture medium at 48 hours (+E), removal of estradiol from the culture medium by replacing the total supernatant by fresh medium at 120 hours (-E), replacing the total supernatant by fresh medium at 168 hours (-M).

the IRF-1-hER complex, this can explain the quick recovery in viability after estradiol removal, since in this new situation there is no longer estradiol to enable IRF-1 activation.

In order to understand if the cell growth inhibited state observed after estradiol removal was not masked by the natural limitations of a batch culture, another experiment with an exchange of medium 3 days after estradiol removal was performed. As can be seen in

Figure 7. Effect upon cell concentration in static culture (A) and viability (B) by a second activation of IRF-1 after one cycle of addition and removal of estradiol (regulated/non-producer clone). ▲ Addition to a final concentration of 100 nM estradiol at 48h (+E_a), removal of estradiol from the culture medium by replacing the total supernatant by fresh medium at 120 hours (-E_a), replacing the total supernatant by fresh medium and addition to a final concentration of 100 nM estradiol at 168 hours (-M+E_b).

Figure 6, cells started to grow again when nutrients were replenished and waste products removed from the medium.

Thus, it can be concluded that estradiol addition initiates a cascade of events leading to growth inhibition and eventually to cell death; estradiol removal stops this cascade, clearly indicating that cell growth inhibition can be reversed.

Since long periods of cell growth inhibition are desired, the cycling hypothesis i.e., that the cells would respond in the same way to a new addition of estradiol

was tested (Figure 7). After one cycle of addition and removal of estradiol with fresh medium replacement, a new activation of IRF-1 due to estradiol addition again led to cell growth inhibition and drop in viability (Figure 7), in a similar pattern to what was described above (Figure 4). This observation shows that the genetic construct used in this study is stable and that by cycling estradiol addition and removal it is possible to regulate cell growth maintaining cell viability. This observation was confirmed by using perfusion cultures for periods longer than one month (data not shown).

From the experiments above it is also possible to realise that the effect of IRF-1 activation upon the general state of the cell (growth, viability, physiology and productivity) is complex and its causes are not well known. It is clear that the activation of IRF-1 causes a disturbance of cell growth; thus, the effects upon the metabolic state after estradiol addition were also studied. The specific glucose uptake and specific lactate production rates of the clones were determined (internal controls in the absence of estradiol were also performed for comparison). Calculations were performed in the same time frames (in the range from 48 to 120 hours after inoculation). Both regulated clones (producer and non-producer) have similar specific glucose uptake in the absence of estradiol (5.9 ± 0.4 and 5.1 ± 1.2 nmol 10^6 cells min^{-1} , respectively); a sharp increase in specific glucose uptake rate was observed for both clones: two fold for the regulated/non-producer clone (11.8 ± 1.6 nmol 10^6 cells min^{-1}) and three fold for the regulated/Factor VII producer clone (18.9 ± 3.7 nmol 10^6 cells min^{-1}). Lactate production rates also increased after cell growth inhibition in the same magnitude of glucose consumption rates, thus maintaining a similar glucose to lactate yield. In all experiments previously described glucose concentration was quantified, confirming that there was no glucose starvation during the batch cultivations (data not shown). It is clear that normal cell populations alter their metabolism and growth rates in response to IRF-1 expression, clearly disturbing the cell metabolic activity. The reason why this variation occurred is not clearly understood.

Many mitogenic stimuli are known to increase the transport of glucose into cells. Some of these include hormones like insulin, growth factors and viral transformation. For this reason, possible side effects over metabolic activity due to estradiol or its solvent were also checked. Estradiol was added to the non-regulated clone, confirming that it has no effect on glucose up-

take and lactate production rates (data not shown). Ethanol did not lead to an increase in glucose uptake, when it was singly added to the culture of the regulated clone. Amino acid measurements were also performed for the experiments described in Figure 4, showing that the sharp decrease in cell viability was not due to any amino acid depletion during the batch runs (data not shown).

Conclusions

When estradiol is added to the culture medium leading to the activation of the constitutively expressed IRF-1-estrogen receptor fusion protein (IRF-1-hER) an effective and rapid cell growth inhibition occurs within 24 hours, but a sharp decrease in cell viability is evident 2 to 3 days after estradiol addition. Although an increase of cell metabolic activity is observed after IRF-1 activation, there is no significant alteration on Factor VII specific productivity.

Furthermore, the manipulation of the culture conditions allows the use of IRF-1 activation as an effective system to control cell proliferation:

IRF-1 activation is reversible and cyclic: when the time of contact between the cells and estradiol is reduced, cell viability increases, achieving similar values to those obtained if no estradiol is added. During this recovery phase, the cells pass two different phases: first a stationary phase extension where cell growth is still inhibited, followed by an increase of cell concentration. This reversible pattern can be repeated for an extended period when estradiol addition and removal are repeated, with similar results being obtained. It is possible to modulate the IRF-1 effect by manipulating cycles of addition/removal of estradiol.

Cell growth inhibition is dependent on estradiol concentration: below 100 nM of estradiol, IRF-1 activation always led to cell growth inhibition, but its extent is significantly dependent upon the estradiol concentration, cell growth inhibition being delayed when the estradiol concentration decreases. All estradiol concentrations above 100 nM led to similar results. The viability decrease was similar in all cases.

Cell growth inhibition is dependent on cell growth phase: a delay in the activation of the IRF-1-hER system by addition of estradiol at later phases of growth is apparent; this can be due to higher cell concentration, since the amount of estradiol per cell is decreased. A clear cell growth inhibition is observed when IRF-1 is activated in the beginning of the exponential phase

(48 hours after inoculation). Cell growth inhibition is independent on clone and culture system. Cell growth inhibition is not due to estradiol solvent (ethanol) or residual estradiol concentration present in the serum and is also independent on serum concentration.

The optimal conditions for the application of this approach were determined: manipulating cycles of addition of estradiol (at the beginning of the exponential phase with a final concentration of 100 nM) followed, after 72 hours, by the total removal of estradiol leads to an effective system for cell growth regulation. Aiming at a broader application, studies will be performed using perfusion system in order to maintain cell density and to easily manipulate cycles of estradiol addition/removal.

This system represents one step towards the maintenance of cells in a tightly controlled and narrow range of proliferation inhibition.

Acknowledgements

The authors are grateful to Dr. Leif Kongerslev from Novo Nordisk for providing the Factor VII producer BHK cell clone and the Factor VII ELISA kit and to Ms. Maria do Rosário Clemente from IBET/ITQB for technical support. The authors acknowledge and appreciate the financial support received from the European Commission (BIO4-CT95-0291) and from Fundação para a Ciência e Tecnologia - Portugal (FMRH/BIC/1788/95 and BIO1117/95).

References

- Ahluwalia BS, Westney LS & Rajguru SU (1995) Alcohol inhibits cell mitosis in G2-M phase in cell cycle in a human lymphocytes in vitro study. *Alcohol* 12 (6): 589–592.
- Bajaj SP, Rapaport SI & Brown SF (1981) Isolation and characterization of human factor VII. Activation of factor VII by factor Xa. *J Biol Chem*, 256(1): 253–259.
- Broze GJ Jr & Majerus PW (1980) Purification and properties of human coagulation factor VII. *J Biol Chem*, 255 (4): 1242–1247.
- Gothelf Y, Raber J, Chen L, Schattner A, Chebath J & Revel M (1991) Terminal differentiation of myeloleukemic M1 cells induced by IL-6: role of endogenous interferon. *Lymphokine Cytokine Res* 10 (5): 369–375.
- Harada H, Fujita T, Miyamoto M, Kimura T, Maruyama M, Furia A, Miyata T & Taniguchi T (1989) Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. *Cell* 58: 729–739.
- Harada H, Takahashi E-I, Itoh S, Harada K, Hori T-A & Taniguchi T (1994) Structure and regulation of the human interferon regulatory factor 1 (IRF-1) and IRF-2 genes: implications for the gene network in the interferon system. *Mol Cell Biol* 14 (2): 1500–1509.
- Kasajima N & Tsutsui T (1990) Cytotoxicity of ethyl alcohol on cultured mammalian cells. *Shigaku* 78 (1): 83–88.
- Kirchhoff S, Schaper F & Hauser H (1993) Interferon regulatory factor 1 (IRF-1) mediates cell growth inhibition by transactivation of downstream target genes. *Nucleic Acids Res* 21: 2881–2889.
- Kirchhoff S, Koromilas A, Schaper F, Grashoof M, Sonenberg N & Hauser H (1995) IRF-1 induced cell growth inhibition and interferon induction requires the activity of the protein kinase PKR. *Oncogene* 11: 439–445.
- Kirchhoff S, Kröger A, Cruz H, Tümmler M, Schaper F, Köster M & Hauser H (1996) Regulation of cell growth by IRF-1 in BHK-21 cells. *Cytotechnology* 22: 147–156.
- Kimura T, Katsutoshi N, Penninger J, Kitagawa M, Harada H, Matsuyama T, Tanaka N, Kamijo R, Vilcek J, Mak T & Taniguchi T (1994) Involvement of the IRF-1 transcription factor in antiviral responses to interferons. *Science* 264: 1921–1924.
- Koromilas AE, Roy S, Barber GN, Katze MG & Sonenberg N (1992) Malignant transformation by a mutant of the IFN-inducible ds-RNA-dependent protein kinase. *Science* 257: 1685–1689.
- Köster M, Kirchhoff S, Schaper F & Hauser H (1995) Proliferation control of mammalian cells by the tumor suppressor IRF-1. *Cytotechnology* 18: 67–75.
- Lengyel P (1993) Tumor-suppressor genes: news about the interferon connection. *Proc Natl Acad Sci USA* 90: 5893–5895.
- Moreira JL, Alves PM, Aunins JG & Carrondo MJT (1994) Changes in animal cell natural aggregates in suspended batch cultures. *Appl Microbiol Biotechnol* 41: 203–209.
- Pellegrine S & Schindler C (1993) Early events in the signalling by interferons. *TIBS* 18: 338–342.
- Tanaka T, Ishihara M, Kitagawa M, Harada H, Kimura T, Matsuyama T, Lamphier MS, Aizawa S, Mak T & Taniguchi T (1994) Cellular commitment to oncogene-induced transformation or apoptosis is dependent on the transcription factor IRF-1. *Cell* 77: 829–839.
- Watanabe N, Sakakibara J, Hovanessian A, Taniguchi T & Fujita T (1991) Activation of IFN- β promoter element by IRF-1 requires a posttranscriptional event in addition to IRF-1 synthesis. *Nucleic Acids res* 16: 4421–4428.
- Wigler M, Silverstein S, Lee L-S, Pellicer A, Cheng Y & Axel R (1977) Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. *Cell* 11(1): 223–232.
- Wilman CL, Sever CE, Pallavicini MG, Harada H, Tanaka N, Slovak ML, Yamamoto H, Harada K, Meeker TC, List AF & Taniguchi T (1993) Deletion of IRF-1, mapping to chromosome 5q31.1, in human leukemia and preleukemic myelodysplasia. *Science* 259: 968–971.