A cell culture model to detect IGF receptor expression during the cell proliferation cycle

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ABSTRACT

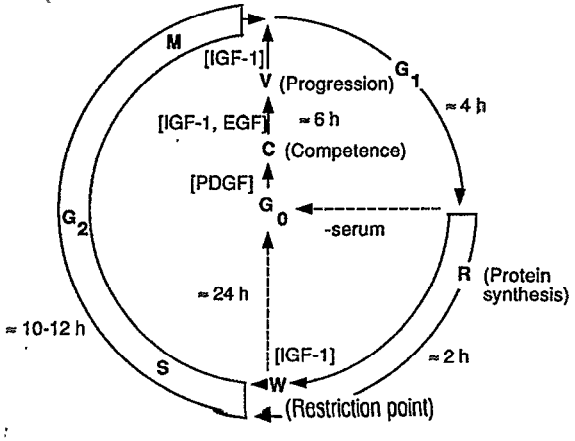
In keeping with the evident requirement of insulin-like growth factor I (IGF-I) for normal growth in livestock, IGF-I is required for the normal proliferation of many non-transformed cells in tissue culture. The timing of IGF-I action is believed to be early in proliferation and/or prior to DNA synthesis. Since IGF-I acts on the Type I and Type II IGF receptors, and on the insulin receptor, it is possible that one or all of these receptors are proliferation regulated, such that they are expressed mainly at a certain stage(s) in the cell proliferation cycle. Regulation might occur at the level of the receptor or at the mRNA level. Several cell types have been chosen to test this model. In the experiments reported here, Norden Lab Feline Kidney (NLFK) cells were grown on coverslips and synchronized using a starve/feed regimen. Coverslips fixed at various times after refeeding were probed *in situ* for the presence of actin RNA and for IGF Type I receptor RNA. Whereas actin RNA levels increased and declined during the proliferation cycle in agreement with earlier reports, Type I receptor RNA levels were undetectable at the times sampled. Possible reasons for this are discussed.

Keywords: cell proliferation cycle, IGF receptor, actin.

INTRODUCTION

Insulin-like growth factor I (IGF-I) is required to achieve growth potential in mammals (Behringer *et al*, 1990), however, a model which directly relates IGF-I action to growth in livestock remains elusive. However, certain mammalian cell culture lines as well as non-transformed diploid cells (Chen and Rabinovitch, 1989; reviewed by Denhardt *et al*, 1986) require IGF-I for normal proliferation. More specifically, IGF-I is required at least for certain cell types to progress through DNA synthesis *in vitro* (Figure 1; Riddle *et al*, 1979; Riddle and Pardee, 1980). Cultured cells can also produce IGF-I (Clemmons *et al*, 1981; Bornfeldt *et al*, 1990), as can *in vivo* regenerating muscle tissue during the proliferation and differentiation of satellite cells (Levinovitz *et al*, 1992).

FIGURE 1: Mammalian (3T3) cell proliferation cycle (adapted from Denhardt et al, 1986).



At this time, the IGF type I and II receptors and the insulin receptor are the only confirmed sites for stimulation by IGF-I or the related peptide IGF-II (reviewed by Rutter, 1991). IGF Type I and insulin receptors have very similar genes and show up to 10% functional cross reactivity with the opposing growth factor (Massagué and Czech,1982; Ullrich *et al*, 1986; reviewed by Rutter, 1991). It is not clear whether a cellular response to insulin or IGF is due to changes in the level, or the activity, of one or both receptors.

Type I receptor binding potential drops at least five-fold during postnatal growth in rats (Werner *et al*, 1989; Alexandrides *et al*, 1989) and possibly in sheep (Oldham, in the press). Receptor RNA levels increase and fall during the rapid proliferation and differentiation of older rat muscle tissue responding to ischemia or snake venom (Levinovitz *et al*, 1992). Apparent IGF Type I receptor binding has been observed to decline with age *in vitro* (detected as a requirement for more IGF-I to progress through the cell cycle by aging diploid fibroblasts; Chen and Rabinovitch, 1989).

Based on the above, it is therefore possible that in a stem cell lineage, IGF receptors function only in daughter cells capable of division, as opposed to terminally differentiated daughter cells which have lost the ability to divide. We would like to test this possibility.

To test our techniques we have examined a kidney cell line undergoing a typical proliferation cycle based on the expression of the ß-actin gene; and probed for the presence of IGF Type I receptor RNA and insulin receptor RNA using moderate stringency *in situ* hybridization (ISH). Since exogenous IGF-I - and thus a receptor - is often required for proliferation *in vitro*, a cell culture model is more likely to turn up an interpretable response than tissues, for examining the degree and timing of expression of genes during a cell proliferation cycle.

MATERIALS AND METHODS

Cell culture

A Norden Laboratory Feline Kidney (NLFK) continuous cell-line was provided by Pittman Moore, N.Z. Ltd. (passage 7 of a mycoplasm-free line). Cells stored in liquid nitrogen were cultured in Dulbecco's Modified Eagle's Medium with 10% fetal calf serum (FCS) using standard techniques. Cells were seeded onto 13 mm NUNC Thermonox coverslips (#174950) in wells of a 24 well tissue culture plate (Falcon). To partially synchronize the cells, subconfluent cultures were starved in MEM with 0.5% fetal calf serum (FCS) for two days, then stimulated with the addition of FCS to 10% (O'Brien et al, 1990). Prior to serum addition and at various times following addition several coverslips were removed and fixed immediately in PBS buffered 10% formalin for 15 minutes, transferred to 70% ethanol and 100% ethanol for 10 minutes each, and air dried. Coverslips were incubated at 50°C overnight prior to further manipulation.

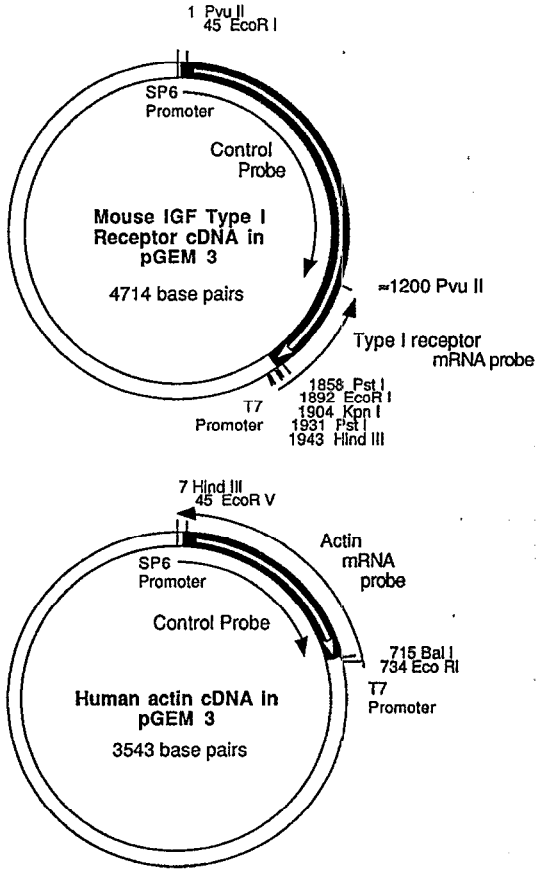
Probes

A cDNA fragment encoding the ß subunit of the mouse Type I IGF receptor was obtained from André Bernards and subcloned as an *EcoRI* fragment of roughly 2 Kb in a pGem3 vector (Promega; Figure 2, Top). The similarity of this fragment with the human and rat Type I receptor sequences was confirmed by sequencing approximately 100 base pairs at either end of the insert and performing FASTA (W.R. Pearson; release 1.6) and TBLASTN (Altshul *et al*, 1990) DNA homology searches of receptor sequences obtained from the EMBL database. For use as a riboprobe, the Type I receptor insert was restricted with PvuII, which cuts once at the 3' end of the predicted coding region. Riboprobes were synthesized using 35S-UTP (Molenaar *et al*, 1992), with the S probe constituting the antisense probe for ovine receptor mRNA, and the T probe functioning as a control for non-specific labelling. A cellular ß-actin mRNA 35S-UTP riboprobe (Figure 2 Bottom; Gunning *et al*, 1983; constructed by Wilkins et al, 1989) was used to detect steady-state levels of cellular actin RNA during the proliferation cycle, since ß-actin mRNA levels are elevated in proliferating cells (Riddle *et al*, 1979,1980; Denhardt *et al*, 1986).

In situ hybridization (ISH)

The procedure for tissue ISH described by Molenaar *et al*, (1992) was used. For each time point two coverslips were analysed: one for actin mRNA and one for Type I receptor mRNA. Coverslips with fixed cells were mounted on slides (face up) and divided in two with rubber cement. To each half of one coverslip a 50% formamide/SSC hybridization solution containing either 400,000 cpm of antisense actin mRNA riboprobe or a sense actin RNA control probe was applied. To one half of the second coverslip 400,000 cpm of antisense Type I receptor mRNA probe in hybridization solution was applied, while 400,000 cpm of (sense) receptor control probe was applied to the other half. The probes hybridized 18 hours at 40°C (which was predicted to allow cross hybridization of both insulin and IGF type I receptor RNA). Coverslips were then rinsed extensively in SSC buffers to remove excess

FIGURE 2: Actin cDNA (Gunning *et al*, 1983) and mouse IGF Type 1 receptor ß subunit cDNA fragment (donated by André Bernards), cloned in pGEM3 vectors. The white arrows inside the black circles represent the cDNAs' orientations and limits. Outside arrows indicate probe orientations and limits.



probe, treated with RNAase to remove non-specifically hybridized probe, rinsed extensively again in SSC buffers, dehydrated in ethanol and dried.

Analysis

Following removal of the rubber cement, the coverslips were exposed for several days in contact with x-ray film (Kodak X-Omat-AR 5). After several such exposures, cell nuclei were counted in 12 random areas of each coverslip to estimate cell numbers at each timepoint, using dark field optics. The coverslips were then coated with Ilford K5 autoradiographic emulsion and left at 4°C for several weeks. Following development, slides were stained with haematoxylin and eosin and a second coverslip mounted on top.

Developed x-ray films were scanned with a Molecular Dynamics Personal lazer densitometer to produce TIFF images. The images were quantitated using the Application IMAGE 1.43 (NIH; Ord; Hodges *et al* this issue), and photographed with a Polaroid Quick Print VI-350 Video Printer (provided by New Zealand Lotteries Grant #022365).

RESULTS

The synchronization of these cells was successful as judged by the rise and fall of actin mRNA levels (Figure 3).

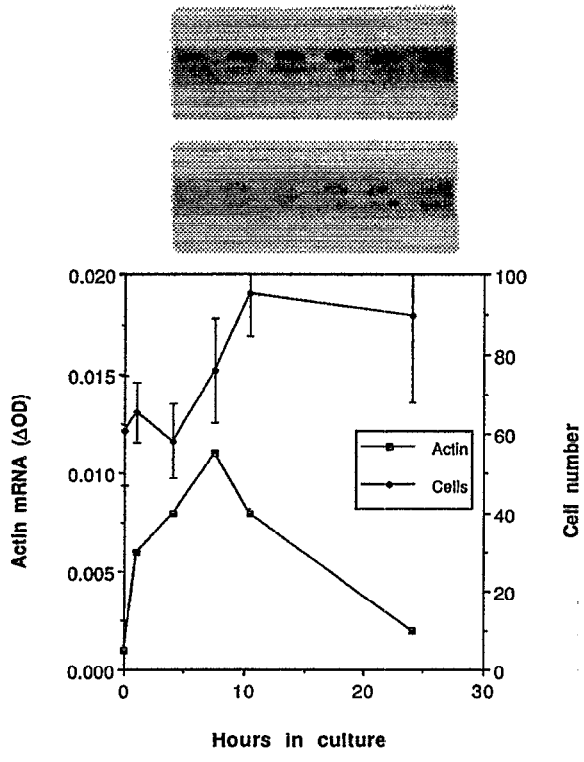
The levels of actin mRNA rose within an hour of addition of growth factors (FCS) to a peak at 7.5 hours, followed by a decline to the levels seen in the starved cells (t = 0).

Since the cells were only monitored over an 18-24 hour period it was of interest to know whether the actin levels were simply correlated with cells adhering to the coverslips, since it is well known that mitotic cells can fall off during routine handling of cell cultures. As shown in Figure 3, after a four hour pause the average cell number detected by counting nuclei with dark field microscopy nearly doubled, indicating that many of the cells had undergone mitosis.

When examined using image analysis or microscopically, the expression of actin mRNA was not homogeneous in this culture, with numerous spots of high silver grain density often evident over clusters of cells (not shown). Since cell clusters present on the control side of the coverslip were not associated with high silver grain density, this result is not artifactual, and suggests that most proliferation is occurring in "foci", as is often seen with immortal cell types such as NLFK cells.

In contrast, the cells on coverslips probed for Type I receptor mRNA (Figure 3, middle) yielded consistently low signals, always at or below the signal of the Type I control RNA probe or the actin control RNA probe. The distribution of signal here was also non-homogeneous, but high silver grain density was not localized to clusters of cells, and was in both control and experimental halves of the coverslip, which indicated non-specific binding. There was an increase in signal relative to the pre-feed control in the Type I receptor mRNA-probed sectors, but because the signal was so low, it is inappropriate to comment further.

FIGURE 3: **Top:** X-ray film images of ^{35}S-UTP labelled actin RNA probe bound to synchronized NLFK cells on coverslips. The top half was probed for actin mRNA, while the bottom half was probed with the actin sense RNA control. Exposure time was three days. **Middle:** X-ray film images of 35S-UTP labelled IGF Type I receptor RNA probe bound to synchronized NLFK cells on coverslips. The bottom half was probed for IGF Type I receptor mRNA, while the top half was probed with the actin sense RNA control. Exposure time was seven days, and computer enhancement of the x-ray film image was required to visualize the extremely low binding. **Bottom (graph):** Relative actin mRNA levels and cell numbers during synchronous growth of NLFK cells. RNA levels are given as ΔOD (optical density measured as image gray level) of bound probe, minus the OD of non-specifically bound probe), while cell numbers (per sq.mm.) were an average of five separate nuclei counts taken at random coordinates from each coverslip.



DISCUSSION

We have examined a cell culture model for determining the presence of IGF Type I receptor gene expression in NLFK cells at the mRNA level using *in situ* hybridization. While the model itself is valid based on the detection of actin gene expression using a human ß-actin cDNA as a marker of proliferation, we were unable to detect significant levels of Type I receptor mRNA above the background of non-specific hybridization. There are a number of reasons why this might be the case.

First, our sampling may have missed a short burst of Type I receptor expression during the proliferation cycle.

Second, the probe might not be sufficiently homologous to either Type I or insulin receptor RNA to hybridize at the stringency tested. Unfortunately an ovine Type I receptor cDNA for probing is not currently available, although a bovine ß-subunit fragment from MDBK cells has been sequenced (Sneyers *et al*, 1991).

Third and most likely, the Type I receptor mRNA was absent or present at very low levels. We would not have detected a signal less than one tenth that of actin mRNA under these conditions. Many receptors are present at extremely low levels. Even where receptors are present in proliferation-capable cells, receptor action may be mediated mainly at the level of signal transduction (reviewed by Rutter, 1991) and not by synthesis or degradation. We have observed similar (unpublished) results - where receptor mRNA probe signal is as low as control probe signal - in several ovine tissue types. When the probes were hydrolysed to less than 200 bases in length, a signal above background became evident in one-day old muscle tissue. However, some of the hybridizing material within the tissue was not degraded by pretreatment with RNAase.

These questions should be resolved when a NIH-3T3 cell line (HIGR1.24) which is capable of both differentiating and undergoing rapid proliferation prior to differentiation, and which overexpresses the Type I receptor (A. Ullrich, Genentech), is tested with this probe, as well as with Type I receptor "oligo" probes homologous only to Type I receptor RNA which we have recently obtained.

It remains possible that a Type I receptor is expressed only by cells capable of proliferation (or specialized cells in kidney which may require it to mediate the IGF-I stimulation of phosphate transport; Caverzasio and Bonjour, 1992). The prediction that the functional loss of the IGF Type I receptor is a marker of the loss of cell division potential may be testable by searching for its presence in terminally differentiated cell types or for its absence in proliferative-capable

(stem) cells. At present this could only be done in differentiating cell cultures using histological ISH or binding studies, since other sensitive techniques cannot distinguish stem cells within a tissue.

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