Pergamon

Biochemical Pharmacology, Vol. 48, No. 9, pp. 1729–1734, 1994.
Elsevier Science Ltd
Printed in Great Britain.

0006-2952(94)00349-1

ANTISENSE OLIGONUCLEOTIDES TO HUMAN LIPOCORTIN-1 INHIBIT GLUCOCORTICOID-INDUCED INHIBITION OF A549 CELL GROWTH AND EICOSANOID RELEASE

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(*Received* 7 *March* 1994; *accepted* 26 *July* 1994)

Abstract—Glucocorticoids actively inhibit the growth of A549 cells by suppressing the release of factors such as prostaglandin E_2 (PGE_2) necessary for their proliferation. This effect is largely mediated through induction of the protein lipocortin-1. We now show that transient transfection of A549 cells with an antisense DNA oligonucleotide targeted to a region coding the unique N-terminal portion of human lipocortin-1 blocks the induction of lipocortin-1 protein following glucocorticoid treatment and completely reverses glucocorticoid-induced suppression of cell proliferation and PGE_2 release. A scrambled oligonucleotide was without effect. Continued culture of A549 cells in the presence of this oligonucleotide results in a sustained increase in cell proliferation and PGE_2 release. This study reinforces the importance of lipocortin-1 as a negative modulator of cell growth and eicosanoid generation in this system.

Key words: lung adenocarcinoma; annexin 1; neutralizing antibody; prostaglandin E_2; protein synthesis

The effects of glucocorticoid action have been shown to be mediated in several systems by the protein lipocortin-1 [reviewed in Ref. 1]. However, the failure to observe an induction of lipocortin-1 following glucocorticoid treatment in some *in vitro* cell system cultures has resulted in confusion over its role in some cells [2, 3]. We have established a model using the A549 human lung adenocarcinoma cell line in which lipocortin-1 is induced on the extracellular cell surface following glucocorticoid treatment [4]. This results in the suppression of cell proliferation secondary to inhibition of the release of factors such as PGE_2† which are necessary to maintain cell growth. The addition of nanomolar amounts of purified recombinant lipocortin-1 (Biogen) to A549 cell cultures mimics the effects of glucocorticoid treatment on both cell growth and PGE_2 release. Moreover, incorporation of a neutralizing monoclonal antibody (1A Biogen) but not a non-neutralizing monoclonal (1B Biogen), in the medium completely reverses this glucocorticoid response [4]. These observations lead us to believe that it is the induced extracellular pool of lipocortin-1 that is crucial for cellular regulation. This pool of lipocortin-1 has hitherto been ignored.

The use of antisense oligonucleotides designed to hybridize with and thus neutralize specific sequences of DNA or RNA has helped to clarify the role of many gene products in cellular function, both *in vivo* and *in vitro* by blocking their production at source. Antisense sequences may be directed against mRNA and are thereby thought to inhibit translation or against DNA and are thereby thought to inhibit both transcription and subsequent translation. However, the precise mechanisms of action of antisense oligonucleotides is far from clear.

We have designed an antisense oligonucleotide complementary to a sequence unique to the N-terminal of lipocortin-1 cDNA in order to investigate the role of endogenously produced lipocortin-1 in the proliferation of A549 cells and to determine how this mediates the effects of glucocorticoids. A scrambled sequence of the same nucleotides was used as an internal control.

MATERIALS AND METHODS

Cell culture. A549 cells (Flow) were maintained in continuous log phase growth in DMEM/F-12 (the Sigma Chemical Co., Poole, U.K.) containing Phenol Red (Sigma) and 10% FCS (Sigma) in T-150 flasks (Greiner). The cells were not allowed to reach confluence at any time as this diminishes their response to growth factors. The cells were routinely checked for the absence of mycoplasma contamination.

Cell proliferation experiments. Subconfluent A549 cells were washed with trypsin (0.05% w/v, Sigma) EDTA (0.02% w/v, Sigma) solution in T-150 flasks for approx. 5 min. The detached cells were seeded into 12-place multi-well plates (Flow Laboratories, Irvine, U.K.) at 5×10^4 cells/mL/well in DMEM/F-12, 10% FCS and incubated overnight. The medium was replaced with DMEM/F-12 (without phenol red) containing dilutions of the appropriate steroid or oligonucleotide. Each culture was

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† Abbreviations: PGE_2, prostaglandin E_2; DMEM, Dulbecco's Modified Eagle's Medium; FCS, foetal calf serum; EIA, enzyme immunoassay.

incubated for 3 days with the medium changed on day 2. The cells were then removed with trypsin/EDTA and counted with a Coulter Multisizer II. Trypan blue exclusion assay was routinely performed—the concentrations of steroid and oligonucleotide used here were not cytotoxic. Each culture was measured in triplicate. All experiments described here were performed under serum free conditions.

Oligonucleotide preparation. We searched within the region of the human DNA coding for the lipocortin-1 N-terminus for a sequence of deoxyribonucleotides which possessed a GC content of at least 60%. We chose bases 83–98 inclusive (5′-CCA AAG GTG GTC CCG G—3′) that fulfilled this criterion. From this a complementary oligonucleotide (3′-GGT TTC CAC CAG GGC C-5′) was constructed as was a scrambled oligonucleotide of the same bases (3′-GTC GTC TAC GAC CGC G-5′) to act as a control. Another anti-sense sequence of bases 21–36 (3′-GGA GTT CGT CCG GAC C-5′) also fulfilled the GC base content criteria but preliminary experiments showed that this oligonucleotide was much less active (data not shown) and it was not pursued further. The oligonucleotides were synthesised by Oswel DNA Service, University of Edinburgh to HPLC purity and were supplied as a 1 μM stock solution in distilled H_2O.

Measurement of PGE_2 production. To provide sufficient material for analysis A549 cells were grown in T-150 flasks under identical conditions to 12-well multi-well plates and steroids, oligonucleotides or vehicle controls were added directly to the flask. After treatment the media was aspirated, centrifuged at 1200 rpm to remove cell debris and stored frozen at −70° until assayed by EIA for PGE_2 using a standard commercially available kit (Amersham).

Measurement of lipocortin-1 protein expression. A549 cells were grown in T-150 flasks as described above. Following treatment with steroid and/or oligonucleotides the culture media was aspirated and the cell monolayer washed with 3 mL of PBS containing 1 mM EDTA, a procedure shown to remove cell surface lipocortin-1 [4]. The remaining monolayer was lysed with 3 mL of PBS containing 10 mM EDTA and 1% Triton-T100, to recover cytosolic proteins. Total protein equivalents were then subjected to SDS–PAGE using Hoefer Mighty Small mini-gels followed by western blotting as described previously [4]. Lipocortin-1 was detected using a specific polyclonal antiserum raised in sheep against the native denatured protein, and quantified by densitometric analysis.

Measurement of lipocortin-1 protein synthesis. A549 cells were seeded at 10×10^4 cells/mL/well in 12-well multiwell plates in DMEM/F12, 10% FCS and equilibrated overnight. The media was then changed to serum free DMEM/F-12 without phenol red and the cells were incubated for a further 24 hr. The media was then replaced with DMEM/F-12 containing 50 nM oligonucleotide/mL/well and incubated for 18 hr. This was then replaced with fresh media containing 250 μCi/mL Trans^{35}S-label (Amersham International, Amersham, U.K.) and fresh oligonucleotide (50 nM) for 3 hr. Dexamethasone was then added to a final concentration of 1 μM and incubated for a further 3 hr after which time the media was discarded and cell surface protein removed with 200 μL PBS containing 1 mM EDTA as for western blotting. This protein extract was then incubated with 10 μL of the immunoprecipitating monoclonal antibody 1B (Biogen) for 3 hr at 4° followed by 50 μL protein A-agarose beads (Sigma) for 1 hr. Antibody bound lipocortin-1 was sedimented by centrifugation at 10,000 g for 5 min and the protein recovered from the beads by washing with 50 mM Tris–HCl, 5 mM EDTA, 1% Triton X-100, pH 8.0 and then subjected to SDS–PAGE on a standard 16 × 20 cm gel (Protean II, Biorad) followed by autoradiography using conventional techniques.

RESULTS

Effect of oligonucleotide treatment on A549 cell growth

When incubated with cell cultures over a 3 day period the antisense oligonucleotide stimulated A549 cell growth in a dose-dependent manner (Fig. 1a) up to a concentration of 20 nM (61.7% above control). A scrambled sequence of the same nucleotides had no significant effect (Fig. 1a). Moreover, cell cultures treated with 5 nM dexamethasone for 3 days were growth inhibited (reduced by 14.8% below control) and this growth inhibition was reversed by the co-incubation of 20 nM antisense oligonucleotide (Fig. 1b) and again the scrambled nucleotide sequence had no effect (Fig. 1b).

Effect of oligonucleotide treatment on lipocortin-1 expression

A549 cells treated with 1 μM dexamethasone for 3 hr expressed an elevated level of lipocortin-1 (208% control as determined by densitometry) in the extracellular cell surface wash as detected by western blotting (Fig. 2). The bands appeared as a doublet of both native (37 kDa) and N-terminally clipped (34 kDa lipocortin-1 and the expression of both bands increased following dexamethasone treatment. This increase in expression was blocked when the cells were pre-treated for 18 hr with the antisense oligonucleotide (50 nM, Fig. 2). However, during this period of treatment basal levels of lipocortin-1 remained unchanged (Fig. 2) indicating that only newly synthesised lipocortin-1 was blocked. Expression of lipocortin-2 and lipocortin-5 remained unchanged (data not shown). Densitometric values were determined from the average of duplicate readings of each band over the background reading using a total grey level densitometry program. The blot and values presented is one typical of three experiments.

Effect of oligonucleotides on lipocortin-1 protein synthesis

Protein synthesis was monitored by incorporating [^{35}S]methionine/cysteine (Translabel, Amersham, U.K.) into the culture media of A549 cells. Following treatment with 1 μM dexamethasone for 3 hr labelled lipocortin-1 appeared in the cell surface wash. The

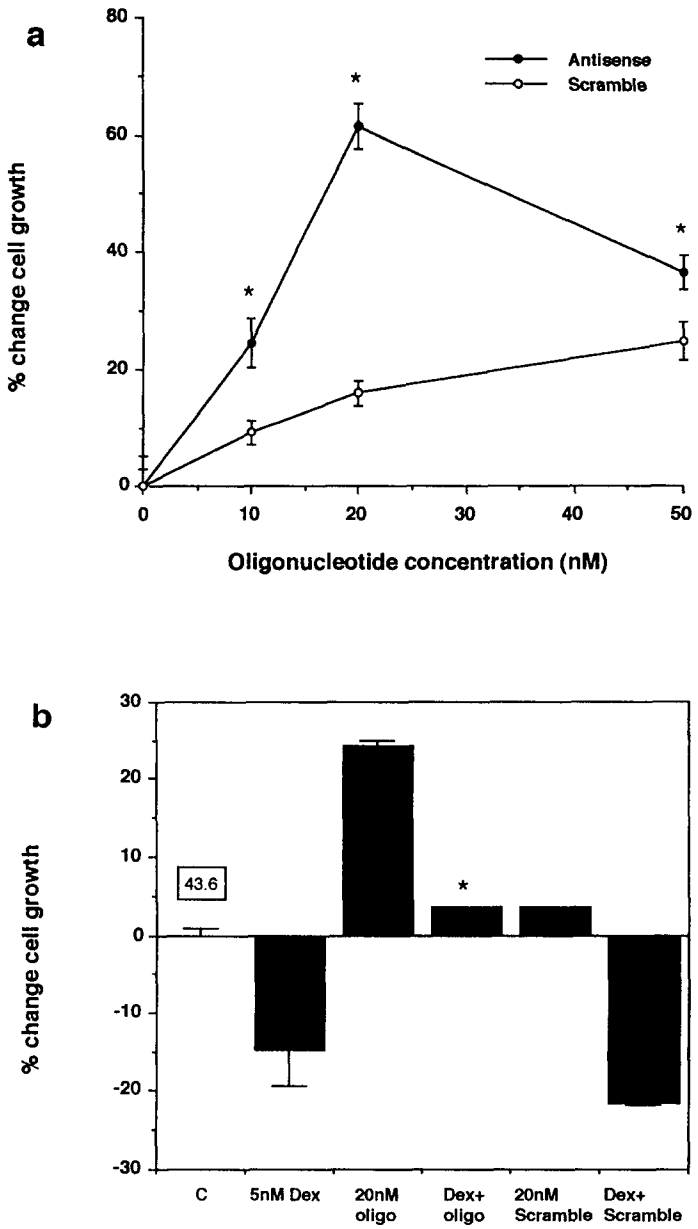

Fig. 1. (a) Antisense oligonucleotide to lipocortin-1 significantly stimulates A549 cell growth in the concentration range 10–50 nM (*). A scrambled sequence of the same oligonucleotides did not significantly effect cell growth. (b) Growth inhibition of 5 nM dexamethasone is significantly reversed by 20 nM oligonucleotide (*) whereas the scrambled sequence has no effect. The actual control cell number is given in the panel ($\times 10^{-4}$). Statistical significance was calculated from raw data but changes in cell growth are plotted as % of control. All cultures were treated for 3 days in DMEM without serum. Each point is the mean of three wells ±SD expressed as a %. Each graph is typical of three experiments where variation in stimulation of cell growth by 20 nM oligonucleotide was between 25 and 60%.

appearance of this pool under dexamethasone stimulation was completely blocked by preincubation with 50 nM antisense oligonucleotide (Fig. 3). This indicates that the appearance of lipocortin-1 at the cell surface is of newly synthesised material and that the antisense oligonucleotide blocks this synthesis which is consistent with the results of the western blotting experiments (Fig. 2).

Effect of oligonucleotide treatment on PGE_2 release

A549 cells release PGE_2 into the culture media (typically around 150 pg/mL in these experiments)

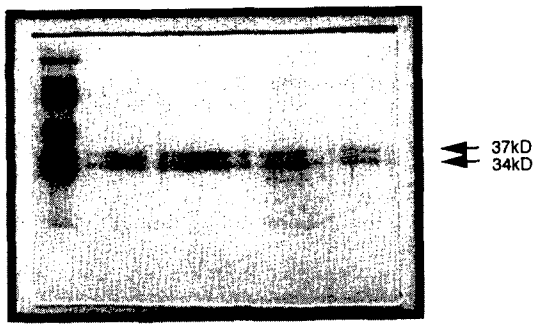

Fig. 2. Treatment with 1 μM dexamethasone for 3 hr significantly increases expression of EDTA cell surface wash of lipocortin-1 in A549 cells as detected by western blotting. This increase is significantly blocked by pre-incubation for 18 hr with 50 nM oligonucleotide. The specific densitometric values in relation to control are: Con 1, Dex 2.2, Oligo 1.1, Dex + Oligo 0.8.

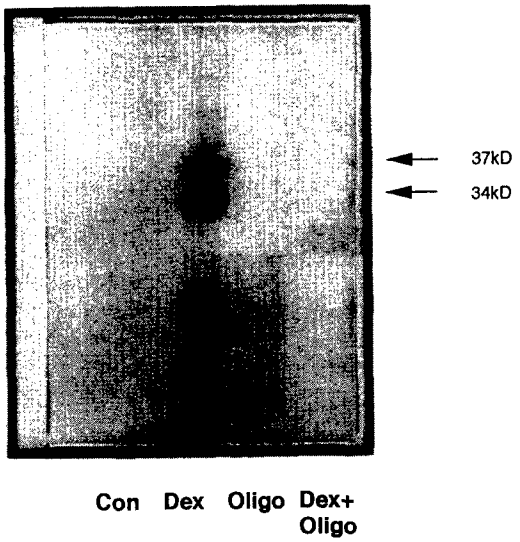

Fig. 3. Treatment with 1 μM dexamethasone for 3 hr significantly increases the synthesis of radiolabelled lipocortin-1 in the EDTA cell surface wash of A549 cells. This effect is completely blocked by pre-treatment with 50 nM oligonucleotide for 18 hr. Each lane is the material recovered from one 12 well multi-well plate following incorporation for 6 hr with 250 μCi Trans-label (Amersham). Cell surface lipocortin-1 was specifically immunoprecipitated as described.

and this release was reduced to 52.4% of control levels after treatment with 5 nM dexamethasone for 24 hr (Fig. 4). However, following a pretreatment for 18 hr, co-incubation of the antisense oligonucleotide (20 nM) reversed this inhibition of PGE_2 release to 83.3% of control levels whereas a scrambled sequence of the same nucleotides was without effect (Fig. 4). The antisense oligonucleotide did not effect basal production of PGE_2 or the inhibitory action of dexamethasone.

DISCUSSION

We report here what we believe to be the first description of activity of an antisense oligonucleotide derived from the N-terminal sequence of lipocortin-1 cDNA. The presence of this oligonucleotide in our A549 cell proliferation model over a 3 day culture period results in a dose-dependent increase in cell growth. Moreover, this oligonucleotide is able to completely reverse the growth inhibitory effects of 5 nM dexamethasone. This observation supports our previous finding where the neutralizing anti lipocortin-1 monoclonal antibody 1A was able to reverse the effect of dexamethasone on cell growth [4]. Furthermore, this reinforces the concept that the growth inhibitory effect of glucocorticoids in A549 cells is mediated through lipocortin-1, which is itself a negative growth modulator.

We have previously shown that expression of lipocortin-1 is increased on the extracellular surface of A549 cells following dexamethasone treatment. This induction is transient and declines within 24 hr. We have also shown that the continued presence of dexamethasone is not required for sustained growth inhibition [4]. These two facts suggest that this transient induction of cell surface lipocortin-1 is able to bring about a sustained inhibition of cell replication perhaps by activating or inhibiting some signaling mechanism. We now show that the antisense oligonucleotide is able to block this dexamethasone-induced pool of lipocortin-1 on the cell surface following a 3 hr pretreatment, but that basal production of the protein remains unaffected during this time. This is perhaps not surprising as the oligonucleotide is designed to hybridize to the cDNA of lipocortin-1 and thus block its expression. Therefore only the expression of newly induced protein will be inhibited and pre-existing protein will remain unaffected subject to its natural turnover.

This is also supported from our observations on [^{35}S]methionine incorporation where treatment with dexamethasone resulted in a large increase of newly synthesised labelled lipocortin-1 immunoprecipitated from the EDTA wash containing cell surface material. Pretreatment with the antisense oligonucleotide completely blocked this induction of the cell surface pool (Fig. 3). In long term cell culture, that is over the 3 day proliferation assay described here where other cellular signals may drive the synthesis of lipocortin-1, then the antisense oligonucleotide can presumably inhibit the endogenous resynthesis of this protein and thus remove the growth restraint under which the cells are normally imposed.

The release of PGE_2 by A549 cells is suppressed by dexamethasone and the addition of PGE_2 to dexamethasone growth-inhibited cultures is able partially to overcome this growth inhibition [4]. These two facts suggest that the autocrine production of factors such as PGE_2 is necessary for cell proliferation and agents that block this process inhibit cell growth. The antisense oligonucleotide to lipocortin-1 is able to reverse dexamethasone induced

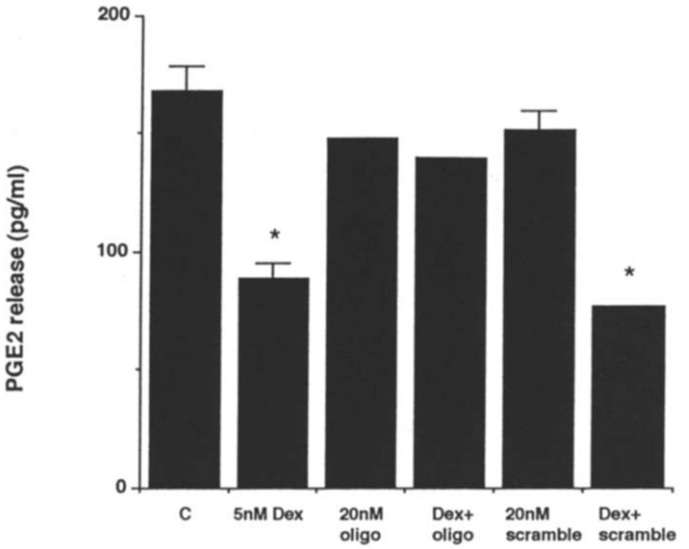

Fig. 4. Treatment with 5 nM dexamethasone for 24 hr significantly inhibits the release of PGE_2 by A549 cells (52.3% control, *). Pre-treatment with 20 nM oligonucleotide for 18 hr does not effect PGE_2 release but significantly reverses dexamethasone suppression of PGE_2 release. The scrambled sequence has no effect on dexamethasone suppression.

suppression of PGE_2 release whereas the scrambled sequence had no effect. This confirms that lipocortin-1 mediates dexamethasone effects on PGE_2 release and that the mechanism of action of the oligonucleotides is ultimately manifested by changes in levels of PGE_2 release. Similarly the use of antisense oligonucleotides to group II PLA_2 RNA has shown that arachidonic acid and PGE_2 release is inhibited following treatment of a macrophage cell line [5].

However, previous reports have apparently shown that the transfection of full length sense or antisense cDNA to lipocortin-1 into a thymic epithelial cell line TEA3A1 produced opposite effects to those seen in our study: in the antisense cells, where the level of lipocortin-1 was reduced by approximately 75%, PGE_2 production was significantly lower and conversely, in sense cells where production of lipocortin-1 was approximately 1.6-fold higher, the cells exhibited increased release of PGE_2 [6]. It was not possible to deduce from the rest of this study whether the effects of glucocorticoids were abrogated in the presence of the antisense DNA. These findings are difficult to reconcile with ours especially in light of the fact that increased levels of lipocortin-1 have been reported in this cell line following treatment with dexamethasone [7]. The authors suggest that inhibition of PGE_2 synthesis by dexamethasone was not mediated through a lipocortin-1 dependent mechanism, however, the cell surface pool of lipocortin-1 was not measured. The data we present here is entirely consistent with our previous observations using neutralizing antibodies on cell growth and PGE_2 release [4].

The concentrations of oligonucleotides used in our study were considerably lower than those reported by other workers to be necessary for blockade of mRNA or protein expression [reviewed in Ref. 8]. Furthermore we have not found it necessary to use modified derivatives in order to achieve an effect. This is possibly because we have deliberately excluded serum from our cultures thus excluding a major source of nucleotidases.

There are many potential mechanisms of action of antisense oligonucleotides including the formation of DNA.DNA, DNA.RNA, RNA.RNA hybrids, triplex DNA structures and interactions with proteins [reviewed in Ref. 9]. Although we have designed an antisense oligonucleotide to specifically hybridize with cDNA other interactions cannot be ruled out. However, the fact that the scrambled sequence of the same nucleotides was without effect would seem to point towards a specific mechanism of action.

The results presented here demonstrate that an antisense oligonucleotide to lipocortin-1 is able to neutralize function in intact cells *in vitro*. The recent observation of oligonucleotide activity *in vivo* [10] offers the possibility that such agents could be used to manipulate lipocortin-1 levels in animal models of disease as well as simple cell synthesis as described here.

Acknowledgements—J.D.C. is supported by the Wellcome Trust and R.J.F. by an endowment from Eli Lilly. We should like to thank Dr J. Browning, Biogen for the provision of monoclonal antibodies 1A and 1B and Oswel, Edinburgh for technical advice on the use of oligonucleotides.

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