Vol. 174, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1991 Pages 287-292

# GROWTH INHIBITION OF HUMAN KERATINOCYTES BY ANTISENSE C-MYC OLIGOMER IS NOT COUPLED TO INDUCTION OF DIFFERENTIATION

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Received December 3, 1990

To study the relationship between cell growth and differentiation in human keratinocytes, we examined the effect of the antisense oligomer of c-myc mRNA. This oligomer is stable in culture medium. A 24 h incubation of cells with 5 μM antisense c-myc oligomer resulted in a 48.2% decrease in c-myc protein and inhibited cell growth by 80.7% compared to the sense c-myc oligomer. In contrast, antisense c-myc oligomer had no effect on differentiation when the population of involucrin-positive cells and cornified envelope formation were used as differentiation markers. These results show that antisense c-myc oligomer inhibits cell growth but does not induce differentiation in normal human keratinocytes. Therefore, cell growth and differentiation are not necessarily coupled in these cells. 

A knowledge of the relationship between cell proliferation and differentiation is essential for understanding biological functions of cells. The epidermal keratinocyte has been a useful model for analyzing cell proliferation and differentiation. Growth inhibition and terminal differentiation are usually observed together in this cell. For example, a shift to high $Ca^{++}$ medium and vitamin $D_3$ induces growth inhibition and terminal differentiation of human keratinocytes (1, 2, 3). However, TGF-β enhances differentiation of these cells under high $Ca^{2+}$ conditions, but inhibits differentiation under low $Ca^{2+}$ conditions, although it arrests cell growth under both high and low $Ca^{2+}$ conditions (4). These data suggest that growth inhibition and terminal differentiation are not necessarily coupled in human keratinocytes. To characterize the independence of growth inhibition and terminal differentiation, we employed an antisense oligomer technique which bypasses the cell surface-mediated events and directly influences the expression of nuclear proteins (5, 6). The c-myc gene is thought to be one of the regulatory genes of proliferation or differentiation in a variety of cells. Recently, it was reported that antisense c-myc oligomer reduces cell growth of BALB/MK cells (7). Therefore, we decided to use this oligomer in this study. We report here that antisense c-myc oligomer inhibits cell proliferation but does not induce differentiation in normal human keratinocytes.

0006-291X/91 $1.50

## Materials and Methods

Oligonucleotides: We used the 15-mer antisense deoxyribonucleotide sequence which is complementary to the 2nd exon initial 15 codons of c-myc mRNA, and the sense oligomer was used as a control (Fig.1)(5). These nucleotides were kindly provided by Earth Pharm. Ltd.(Tokyo). Both oligomers were lyophilized and suspended in Hepes-buffered saline at pH 7.3.

Cell culture: Normal human epidermis was obtained during plastic surgery and keratinocytes were cultured using a modified method of Wille et al. as described previously (1, 8). Second passaged cells were used, and bovine hypothalamic extract was omitted from the culture medium to insure the stability of the oligomer in this experiment.

Oligonucleotide stability: The 5' ends of the oligomers were labeled with $\gamma$-[$^{32}$P]-dCTP (Amersham, UK) using T4 polynucleotide kinase (Takara, Kyoto). Their specific activity was about 2 X$10^8$ cpm/$\mu$g. Next, 5.4 nM labeled oligomers and 5$\mu$M non-labeled oligomers were added to the human keratinocyte culture. The culture medium was harvested at 0, 1, 4, 12 and 24 h and lyophilized. The concentrated medium was suspended in 10 mM Tris-HCl pH 8 containing 1 mM EDTA and boiled to shear the nucleotides. These samples were submitted to urea-containing 10% polyacrylamide gel electrophoresis (PAGE) and analyzed by autoradiography.

Western blot analysis of c-myc protein: Cells incubated for 24 h with 5 $\mu$M sense or antisense oligomers for 24 h were lysed and homogenized in Laemmli sample buffer (9). The protein content was adjusted to equal concentrations as determined by Lowry's method (10). These samples were separated by10% SDS-PAGE. Immunoblotting was performed by using a modification of a standard method (11). Mouse anti-human c-myc antibody (Oncogene Science, Manhasset, NY) and biotinylated horse anti-mouse immunoglobulins (Vector Laboratories Inc., Burlingame, CA) were used in this experiment. The relative intensity of the stained band was measured using a dual-wavelength flying-spot scanner (CS-9000, Shimadzu, Kyoto).

Measurement of cell growth : After the growth period, the cells were stained with 0.2% crystal violet in 0.1 M citric acid. The stained area was measured using an image processor system (4). Cell growth calculated by this method was consistent with the conventional method of cell counting using a hemocytometer.

Bromodeoxyuridine (BrdU) uptake and [$^3$H]-Thymidine Incorporation : Keratinocytes cultured on chamber slides (Lab Tek, Nunc Inc., Naperville Ill) were treated with 5 $\mu$M of antisense and sense oligomers for 21 h, followed by a 3 h incubation with thymidine-free medium containing

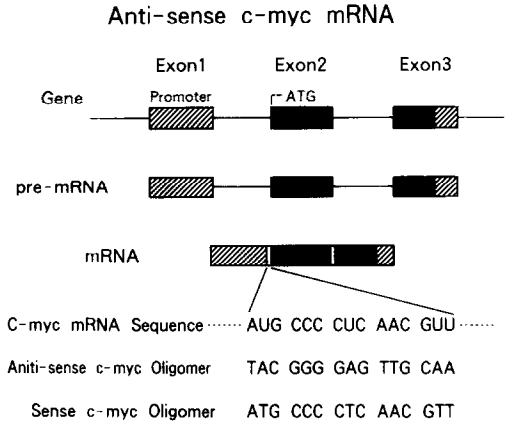



Fig.1. Sequence of sense and antisense oligomers.
We used sense and antisense oligomers complementary to the codons of c-myc from the 1st nucleotide to the 15th exon 2 coding region. They are composed of unmodified deoxyribonucleotides.

400 μM of BrdU. Cells were stained as described previously (2). One thousand nuclei were counted per sample and the percentage of BrdU-positive cells was calculated.
[$^3$H]-thymidine incorporation was measured as follows (2). Keratinocytes in 48-well trays (Falcon, Oxnard, CA) were treated with 1,25$(OH)_2D_3$ and pulse-labeled with 1 mCi of [methyl-$^3$H]-thymidine (25 Ci/mmol, CEA) for 1 h. Cultures were washed 3 times with PBS and twice with 10% (w/v) cold trichloroacetic acid. Cells were solubilized with 1 M NaOH and radioactivities were counted with a scintillation counter. Averages of triplicate measurements were calculated.

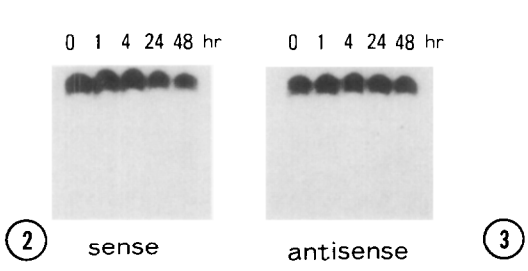
Measurement of differentiation markers: Cells were incubated with antisense or sense oligomers for 48 h. The percentage of involucrin-positive cells was estimated by flow cytometry as described previously (12). The formation of cornified envelope was measured using [$^{35}$S]-methionine-labeled human keratinocytes according to the method of King et al.(13).

## Results

Stability of the antisense and sense oligomers: To determine the stability of antisense and sense oligomers in culture medium, [$^{32}$P]-labeled oligomers were incubated for 48 h in medium free of bovine hypothalamic extract. The autoradiograph showed no degradation product band (Fig. 2). This indicates that these oligomers were stable in this culture medium.

Effect of antisense c-myc oligomer on c-myc protein synthesis: Human keratinocytes were incubated with 5 μM of sense and antisense c-myc oligomers for 24 h and cell extracts were analyzed by Western blotting. Specific binding of anti-human c-myc antibody to the 60 kDa protein was observed in lanes of keratinocyte extracts treated with sense and antisense c-myc oligomers (Fig.3). However, the intensity of this band was markedly weaker in the lane of sample treated with antisense c-myc oligomer. This decrease was estimated to be 48.2% by densitometric analysis.

Effect of antisense c-myc oligomer on the growth of keratinocytes: Human keratinocytes were refed with fresh medium containing various concentrations of antisense or sense oligomers 1 day and 5 days after plating on a 12-well plate (2 x $10^4$/well). The culture was terminated after 8 days and cells were stained with 0.2% crystal violet. Addition of antisense oligomer markedly inhibited human keratinocyte growth, while addition of sense oligomer had no effect compared to the



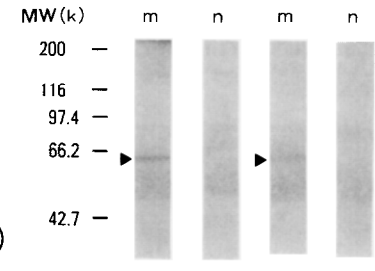
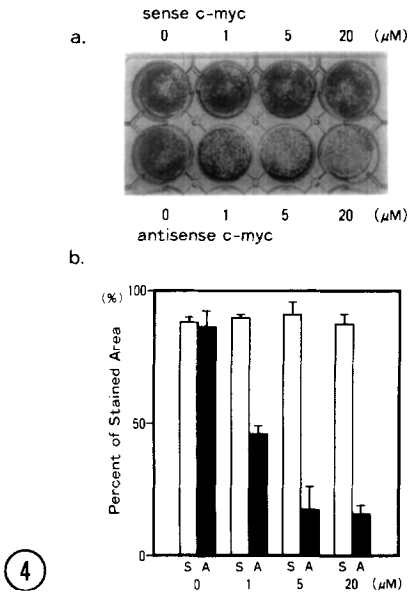




Fig.2. Autoradiographs demonstrating the stability of the 5'-end of labeled sense and antisense c-myc oligomers incubated with normal human keratinocytes.

Fig.3. Immunoblotting of c-myc protein in extracts of human keratinocytes treated with 5 μM sense or antisense c-myc oligomers. m: anti-human c-myc monoclonal antibody, n: control ascites.



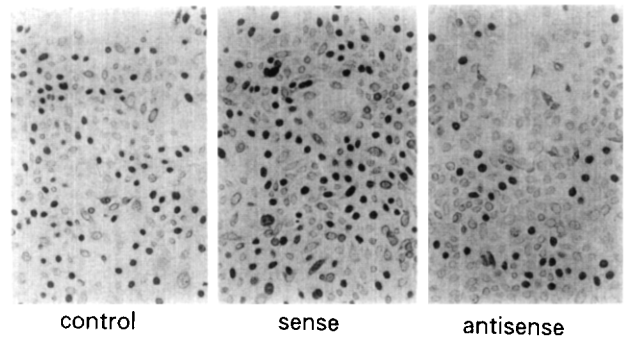




Fig.4. Effect of sense and antisense oligomers on growth of normal human keratinocytes. The detailed procedure is described in Materials and Methods. a: Photograph of the stained plate. Cells in the upper row were treated with sense c-myc oligomer. Cells in the lower row were treated with antisense c-myc oligomer. b: Measurement of stained area relative to total plate area. The open bar indicates the stained area of cells treated with sense c-myc oligomer (S), and the solid bar indicates the stained area of those treated with antisense c-myc oligomer (A).

Fig.5. BrdU uptake by normal human keratinocytes treated with 5 μM sense or antisense c-myc oligomer.

control (Fig.4). Measurement of stained areas showed that cell growth was reduced with 1 μM, 5 μM and 20 μM of antisense oligomer by 48.9%, 80.7% and 82.2%, respectively, compared to the sense oligomer at the same concentration.

Bromodeoxyuridine (BrdU) uptake and [$^3$H]-Thymidine Incorporation : 37% of the nuclei of the untreated control cells were labeled. Cultures treated with 5 μM sense c-myc oligomer had a labeling index of 38%. Antisense c-myc oligomer at 5 μM reduced labeling to 21% (Fig. 5). [$^3$H]-thymidine incorporation was measured 12 h and 24 h after addition of 5 μM sense and anti-sense c-myc oligomer. As shown in table 1, 5 μM anti-sense c-myc oligomer reduced [$^3$H]-thymidine incorporation by 16.3% at 12 h and 43.4% at 24 h compared to sense c-myc oligomer. These results indicate that this antisense c-myc oligomer can inhibit growth of normal human keratinocytes.

Table 1

Time course of inhibition of [$^3$H]-thymidine incorporation by antisense c-myc oligomer

| Time | | 12 h | 24 h |
|---|---|---|---|
| Control | | 1007 ± 29 | 1197 ± 185 |
| Sense | 5μM | 1060 ± 37 | 1076 ± 280 |
| Antisense | 5μM | 887 ± 5 | 587 ± 74 |

The averages of triplicate measurements (DPM ± S.D.) are shown.

Table 2

Effects of sense and antisense c-myc oligomers on keratinocyte differentiation

| Treatment | | Involucrin positive-cells (% of total cells) | Cornified envelope formation (% of detergent-insoluble protein) |
|---|---|---|---|
| Control | | 10.3 ± 3.2 | 6.35 ± 0.50 |
| Sense | 5μM | 10.0 ± 4.6 | 5.71 ± 0.19 |
| | 20μM | 11.3 ± 2.5 | 6.91 ± 0.09 |
| Antisense | 5μM | 9.5 ± 3.0 | 6.26 ± 0.25 |
| | 20μM | 10.3 ± 3.4 | 6.60 ± 0.36 |

Effects of sense and antisense c-myc oligomers on keratinocyte differentiation: Involucrin synthesis was expressed as the percentage of involucrin-positive cells. There was no difference in the percentage of involucrin-positive cells among control cells, antisense, and sense oligomer-treated cells (Table 2). Cornified envelope formation was expressed as the percentage of detergent-insoluble protein compared to total [$^{35}$S]-methionine-labeled protein. There was also no difference in the percentage of detergent-insoluble protein among control cells, antisense, and sense oligomer-treated cells (Table 2). These results indicate that antisense c-myc oligomer does not modulate human keratinocyte differentiation.

## Discussion

This study demonstrates that 1) antisense c-myc oligomer inhibits cell growth but does not induce differentiation in normal human keratinocytes, and 2) cell growth and differentiation are not necessarily coupled in these cells. The use of the antisense oligomer technique is fairly uncommon. Most previous studies have used culture conditions in which both growth inhibition and terminal differentiation were ongoing simultaneously (1, 2, 3). The use of the antisense oligomer technique msde it possible to study growth inhibition and differentiation in human keratinocytes separately. The stability of oligomers has been shown to be important in tissue culture experiments (6). Therefore, in this study, we employed a serum-free culture. The antisense deoxyribonucleotide sequence complementary to c-myc mRNA without residue modification was stable in human keratinocyte culture. Previously, Holt et al. reported HL-60 promyelocytic cells are inhibited by the same antisense oligomer complementary to c-myc mRNA (5). Pietenpol reported that the growth of mouse BALB/MK keratinocytes was inhibited by antisense phosphorothioate oligomer complementary to c-myc (7).

Previous data indicate that suppression of c-myc expression is associated with inhibition of cell proliferation (5, 7). However, the effect of c-myc gene expression on differentiation is controversial. In the mouse erythroleukemia cell line, c-myc gene expression does not induce differentiation (14,15). Inhibition of c-myc gene expression by antisense nucleotides induces differentiation in HL-60 human promyelocytic cells (5), but not in mouse F9 teratocarcinoma (16). The relationship between c-myc oncogene expression and differentiation in U-937 and HL-60 human myeloid cells is dependent on triggers for induction of differentiation (17). Retinoic acid

induced differentiation with reduction of c-myc expression. In contrast, γ-interferon induced differentiation without reduction of c-myc expression. In mouse primary keratinocytes, the relationship between c-myc expression and differentiation is complicated. Calcium-induced differentiation did not suppress c-myc expression, showing persistent c-myc expression irrespective of their proliferative and differentiated state, while TPA-induced differentiation suppressed c-myc expression transciently (18). Thus, the effect of c-myc expression on differentiation seems to depend on cell type and type of triggers for induction of differentiation. The choice of markers and their quantitative measurement should be considered in estimating keratinocyte differentiation. Involucrin is a precursor protein of detergent-insoluble cornified envelope (19). Both involucrin and cornified envelope are thought to be good markers of terminal differentiation of human keratinocytes. Methods for measurement of involucrin-positive cells by flow cytometry and detergent-insoluble protein labeled with [$^{35}$S]-methionine are well established and provide a objective means of quantitative analysis (12, 13).

The finding that growth inhibition and differentiation can occur independently in human keratinocytes has been previously reported (4, 20). Reise and Zhou showed that TGF-β regulates the calcium-induced terminal cell division in the BALB c/MK1 cell independently of the induction of transglutaminase, a marker of keratinocyte differentiation. Taken together with our present results, it seems that growth inhibition and terminal differentiation are closely related but regulated independently in human keratinocytes.

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