Journal of Photoscience (2002), Vol. 9(2), pp. 451–453

Photo-controlled gene expression by fluorescein-labeled antisense oligonucleotides in combination with visible light irradiation

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A new concept of "photo"-antisense method has been evaluated, where the inhibition of gene expression by the conventional antisense method is enhanced by photochemical binding between antisense oligonucleotides conjugated with photo-reactive compound and target mRNA or DNA. Fluorescein labeled oligodeoxyribonucleotides (F-DNA) was delivered to cell nuclei in the encapsulated form in multilamellar lecithin liposomes with neutral charge. F-DNA was previously shown to photo-bind to the complementary stranded DNA, and the delivery system using neutral liposome to be effective in normal human keratinocytes. In the present study, we used human kidney cancer G401.2/6TG.1 cell line to be advantageous in reproducible experiments. p53 was adopted as a target gene since antisense sequence information has been accumulated. The nuclear localization of F-DNA was identified by comparing the fluorescence of F-DNA with that of Hoechst 33258 under fluorescence microscope. After 7hr incubation to accumulate p53 protein induced by UV-B, p53 protein was quantified by Western blot. After 2hrs from F-DNA application, about 30% of cell population incorporated F-DNA in their nuclei with some morphological change possibly due to liposomal toxicity. Irradiation of visible light longer than 400nm from solar simulator at this time enhanced the inhibitory action of antisense F-DNA. The present results suggest that photo-antisense method is promising to control gene expression in time and space dependent manner. Further improvement of F-DNA delivery to cancer cells in the stability and toxicity is in progress.

Key words: photo-antisense, fluorescein, oligonucleotide, p53, UV-B, G401.2/6TG.1

INTRODUCTION

The antisense method have been widely used for inhibiting protein synthesis by suppressing gene expression through complementary binding between antisense oligonucleotide and target mRNA or DNA. In order to enhance and improve the inhibitory effect, the "photo-" antisense method has been proposed in which antisense oligonucleotides are labeled with photoactive fluorescein [1]. Upon visible light irradiation photochemical covalent binding between both strands would be expected, which would result in the enhancement of the antisense effect. The advantages of this method are summarized as follows: 1) enhancement of gene inhibition, 2) identification of intracellular localization by fluorescence microscope which enables the control of the antisense effect depending on the intracellular location of the antisense oligonucleotides, and 3) control of gene inhibition depending on the irradiation timing of visible light.

Similar trial has been reported using psoralen labeled antisense oligonucleotides [2, 3]. Psoralen itself has been known to inhibit gene expression upon UV-A radiation. In combination with the antisense method significant growth inhibition and reduction of mRNA of target genes were observed [3]. While psoralen is a well-known DNA binding dye with preferential crosslinking to specific bases, information about interaction between fluorescein and DNA is rather limited to our knowledge. We expect that under dark condition fluorescein labeled oligodeoxyribonucleotides (F-DNA) bind a target sequence through only hydrogen bonding between complementary strands without any interference from the labeled dye, and that the following irradiation of visible light triggers photochemical binding in addition to hydrogen bonding. From the viewpoint of future

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clinical application, visible light is more profitable in the penetration through the skin compared with UV-A light.

In the previous study we evaluated a possibility of the photo-antisense method using p53 gene as a model target gene in normal human keratinocytes [4]. Keratinocytes have been known to uptake antisense oligonucleotides in their free form [5] or encapsulated form into neutral charged liposomes [1]. Although slight enhancement of gene inhibition was observed upon visible light irradiation, the cellular system has several problems in continuing further experiments. That is, the guaranteed passage degree of keratinocytes is limited only to the fourth culture. Consequently the uniformity of cell nature may be lost and the monetary cost is required for every culture.

In the present study, we adopted human cancer cell line for the reproducible experiments and tried to obtain correlation between nuclear incorporation of F-DNA and the degree of the photo-antisense effect.

MATERIALS AND METHODS

Cell culture. Human kidney cancer cell line, G401.2/6TG.1, (informally called as SB3) was originally derived from a Wilms' tumor patient and established as described previously [6]. The cells are grown in RPMI1640 medium supplemented with 5% Fetal Bovine Serum.

Preparation of F-DNA. F-DNA was encapsulated in multilamellar lecithin liposomes with neutral charge [1]. An antisense oligonucleotide for p53 is 18mer with the sequence of 5'-CGGCTCCTCCATGGCAGT-3'.

Observation of nuclear accumulation of F-DNA. The nuclear localization of F-DNA was identified by comparing the fluorescence of F-DNA with that of Hoechst 33258 under fluorescence microscope. The nuclear uptake ratio was expressed by the number of cells that uptake F-DNA into their nuclei divided by the number of total cells.

UV-B and visible light irradiation. Broad band UV-B was used from fluorescence lamp (FGL-6E; Toshiba, Japan). After irradiation cells were incubated at 37°C under 5% CO_2 atmosphere to allow the expression of p53 gene. Visible light irradiation was from a 500W solar simulator with a cut off filter that passes light whose wavelength is longer than 400nm.

Western blot analysis. Protein extraction was carried out from incubated cells after UV-B irradiation by heating at 103°C in the lysis buffer containing 0.1% SDS, which was followed by the separation with polyacrylamide gel electrophoresis. Proteins transferred to a blotting membrane were sequentially stained with p53 monoclonal antibody (Novocastra Laboratories, U. K.) and anti-mouse IgG peroxidase-linked species-specific whole antibodies (Amersham Pharmacia Biotech, U. K.). Pattern of Western blot were analyzed with an image processing computer software Scion Image (Scion Corporation, U. S. A.).

RESULTS AND DISCUSSION

Uptake of F-DNA into cellular nuclei

Figure 1 shows the uptake ratio plotted against incubation time of cells with F-DNA encapsulated in the neutral charged liposomes. After 15 min incubation, F-DNA has already been accumulated in the cell nuclei about 18%. Nuclear uptake ratio increased with extending the incubation time, followed by the saturation tendency about 30% after 2hr incubation. From this time course, we determined the time of visible light irradiation as 2hrs after the incubation.

It should be noted that F-DNA uptake cells have some morphological changes with rather round shape. An extreme case showed nucleus moved outside the cell, indicating the occurrence of necrosis. This may result from liposomal toxicity due to the constituent lecithin. This will be one of critical issues in the future research.

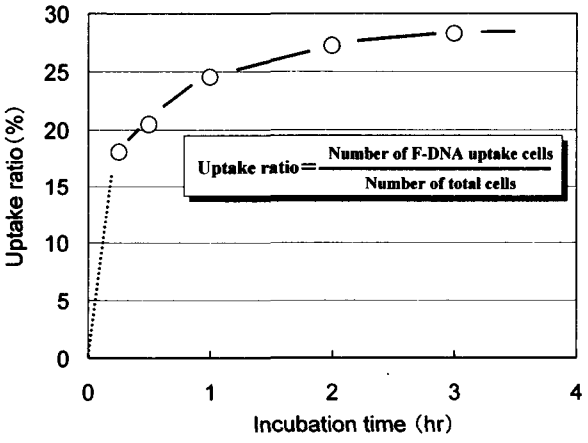

Figure 1. Uptake of F-DNA into cellular nuclei of SB3. The definition of uptake ratio is described in the figure.

Induction of p53 by UV-B radiation. p53 induction in SB3 cells is suppressed to the low level, suggesting the normal status of p53. UV-B irradiation was found to induce p53 significantly. Figure 2 is Western blot patterns showing the induction of p53 with 50J/m^2 UV-B irradiation with the following 7hr incubation at 37°C. Since 7hr incubation exhibited nearly the maximal induction, in the photo-antisense experiments this induction condition was decided to use.

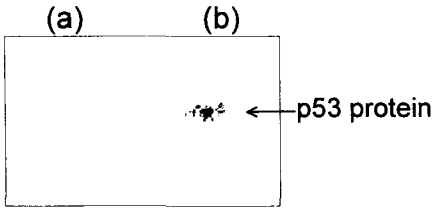

Figure 2. Western blot pattern showing the production of p53 protein after UV-B irradiation.
(a) Without UV-B irradiation (control)
(b) 7hr incubation after UV-B irradiation (50J/m^2)

Effect of visible light irradiation on the inhibition of p53 protein production by F-DNA. The inhibitory effects of F-DNA on the induction of p53 were summarized in Figure 3. The upper panel shows Western blot patterns and the lower panel illustrates the relative band intensity analyzed by the Scion Image software. As shown in the lane (B) p53 protein induction was significantly reduced to about 68% of the induction level by UV-B, indicating the conventional antisense effect. Upon visible light irradiation as shown in the lane (C), further inhibition was achieved. These results demonstrate that the fluorescein labeling technique is promising for the improvement of the antisense method.

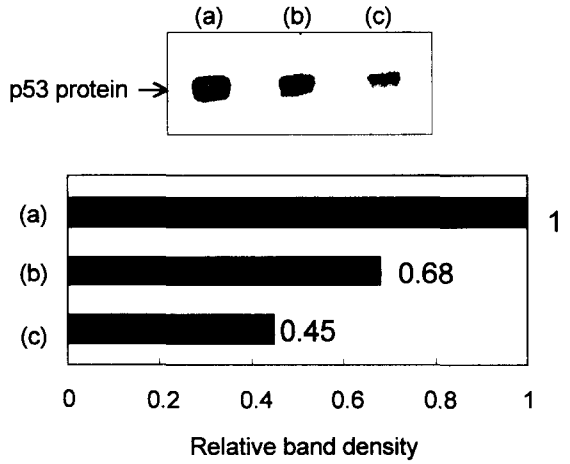

Figure 3. F-DNA inhibition of p53 protein production by UV-B irradiation.
(a) Without F-DNA (control)
(b) Addition of F-DNA without visible light irradiation
(c) Addition of F-DNA with visible light irradiation

Remaining issues to be solved in the future. Although we presented the usefulness of the photo-antisense method to control gene inhibition that can be triggered by photo-irradiation, several problems remain. First, the timing of visible light irradiation should be further optimized by considering the intracellular location of the antisense oligonucleotides. Fluorescein labeling will be very helpful for visualization of the location of antisense oligonucleotides. Second, the delivery system of oligonucleotides into cells should be improved to attain further nuclear uptake of F-DNA. In our case of neutral charged liposomes, the reduction of cellular toxicity and the increase in incorporation efficiency of F-DNA into liposomes will be urgent issues.

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