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ANTISENSE RNA TO THE TYPE I INSULIN-LIKE GROWTH FACTOR RECEPTOR REVERSED THE TRANSFORMED PHENOTYPE OF PC-3 HUMAN PROSTATE CANCER CELL LINE *IN VITRO*

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ABSTRACT

The insulin-like growth factor I receptor (IGF-IR) plays an essential role in the establishment and maintenance of transformed phenotype. Interference with the IGF-IR pathway by antisense causes reversal of the transformed phenotype in many rodent and human tumor cell lines. We stably transfected the PC-3 human prostate cancer cell line with an IGF-IR antisense RNA expression plasmid. The number of IGF-I receptors on the antisense-transfected PC-3 cells was reduced by 40.2% relative to the control-transfected cells. The transfected cells maintained their high expression of IGF-IR antisense RNA for up to one year in selective medium. The reduction in the expression of IGF-IR had no effect on the cell growth in monolayer. The clonogenicity of antisense-transfected cells was 24.7% of the clonogenicity of control-transfected cells in soft agar. There was a good correlation between IGF-IR level and inhibition of transformation in soft agar. These results indicate that reduction of IGF-IR by antisense RNA can reverse the transformed phenotype of human prostate cancer cells.

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Keywords: Type I insulin-like growth factor receptor; Antisense; Transformation; prostate cancer; PC- 3.

INTRODUCTION

The insulin-like growth factor (IGF) axis form an important regulatory system in growth and differentiation of normal and neoplastic cells.[1-4] The IGF system involves a network of molecules that include IGFs (IGF-I and IGF-II), IGF receptors (IGF-IR and IGF-IIR), IGF-binding proteins (IGFBP-1 through -6), and IGFBP proteases.[5-7] The majority of the mitogenic effects of the IGFs appear to be mediated via the type I IGF receptor.[8] The IGF-IR has been shown to play a central role in the mechanism of transformation and metastatic potential.[9-11]

The antisense technology has been extensively used *in vitro* and *in vivo* as a tool to study the regulatory

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mechanisms in biologic processes and as a potential therapeutic agent in cancer, viral infections, and genetic disorders.[12] A series of experiments based on antisense strategies against the IGF-IR RNA have shown that a decrease in the number of IGF-IRs causes a reversal of the transformed phenotype, as measured by colony formation in soft agar.[13-15] The reduction in the number of IGF-IRs has been achieved by two basic approaches: (a) stable transfection of a plasmid expressing an antisense RNA to the IGF-I receptor RNA; or (b) incubation of cells with antisense oligodeoxynucleotides against the IGF-IR RNA.[16-18]

Prostate cancer is the second leading cause of death in men in many regions of the developed world.[19] While the importance of the IGF axis in the normal prostate is unknown, there is evidence to support the hypothesis that signaling through the IGF-IR appears to be critical for malignant prostate cell growth *in vitro* and blocking IGF-IR signaling inhibits growth of human prostate cancer cell lines.[20]

The PC-3 prostate cancer cell line was originally isolated from a metastasis of a human prostatic adenocarcinoma to bone.[22] Receptor studies have demonstrated the presence of specific binding sites for IGF-I on the PC-3 cells.[23] In this work, PC-3 cells were stably transfected with a new antisense IGF-IR construct. This construct has a 696bp fragment of IGF-IR cDNA in the antisense direction relative to the CMV promoter. The antisense-transfected PC-3 cells have maintained their high expression of IGF-IR antisense RNA for up to one year in selective medium. The reduction in the number of IGF-I receptors reduced the number of colonies on soft agar significantly. However, the growth of PC-3 cells was not affected by the expressing IGF-IR antisense RNA and the doubling time did not change.

MATERIAL AND METHODS

Cell culture

The PC-3 human prostate cancer cell line was obtained from American Type Culture Collection (Rockville, MD). Cells were passaged in RPMI-1640 (GIBCO/BRL, Paisley, Scotland) medium supplemented with 10% Fetal Bovine Serum (FBS, GIBCO/BRL, Paisley, Scotland), 120 units/mL of penicillin and 200 mg/mL of streptomycin. Cell viability was determined by trypan blue dye exclusion test. The cells were incubated at 37°C, 5% CO_2, and full humidity. Cells from subconfluent cultures were used for all assays.

Growth in monolayer

The antisense- or control-transfected cells (1.9×10^4) were plated on each well of 24-well plates in RPMI-1640 medium supplemented with 10% FBS. Every two days, half of the medium was replaced by fresh medium. The cells from triplicate wells were removed by trypsin-EDTA every day for 14 days. Trypsin-EDTA (0.25%, 1mM) was added to the cells, after 90 seconds it was removed and the cells were incubated in incubator for 15 min. Cells were collected and counted in a hemocytometer. The doubling time was calculated from growth curve.

Colony formation assay

1×10^4 of antisense-transfected cells or 1250 of control-transfected cells were cultured in RPMI-1640 medium supplemented with 0.3% agar (Difco, Michigan, USA) and 10% FBS in the final volume of 0.4 mL in each well of a 24-well plates (NUNC, Roskilde, Denmark). The cells were incubated for 10 days at 37°C and full humidity with 5% CO_2. Colonies were stained with Wright stain and those containing 50 or more cells were counted. The plating efficiency (PE) was defined as
PE= (Number of colonies x 100)/Number of cells plated

Construction of the plasmid pIGF-IRAS

The human IGF-IR cDNA fragment containing 696 bp (from nucleotide position 42 in exon 1 to nucleotide position 738 in exon 3) was cloned into the BamHI/PstI sites of the pBK-CMV vector. The vector contains the cytomegalovirus promoter that derives the expression of the insert in a constitutive manner. The SV40 polyadenylation signal sequence and a neomycin resistance gene under control of the CMV promoter are present at the 5' end of the IGF-IR fragment (Fig. 1). T3 and T7 promoters are present at the 3' and 5' ends of insert, respectively. After cloning and transformation, the white colonies were digested with BamHI and PstI enzymes. The white colonies containing a 696bp fragment of the IGF-IR cDNA was chosen. The presence of the IGF-IR cDNA insert in the antisense direction was confirmed by sequencing. DNA was amplified by PCR using universal vector primers T7 or T3 for 25 cycles of 98°C for 30 sec, 50°C for 15 sec, and 60°C for 4 min.[24]

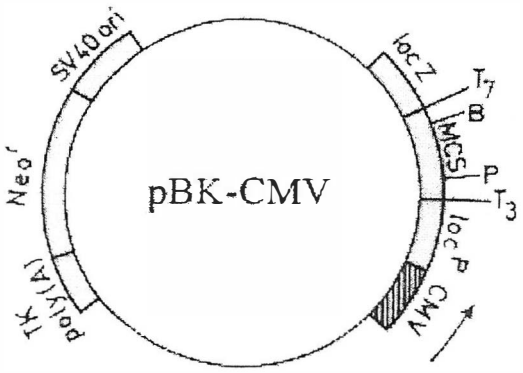
The sequence of T7 primer: (3´-CGGGATATCACTCAGCATAATG-5)

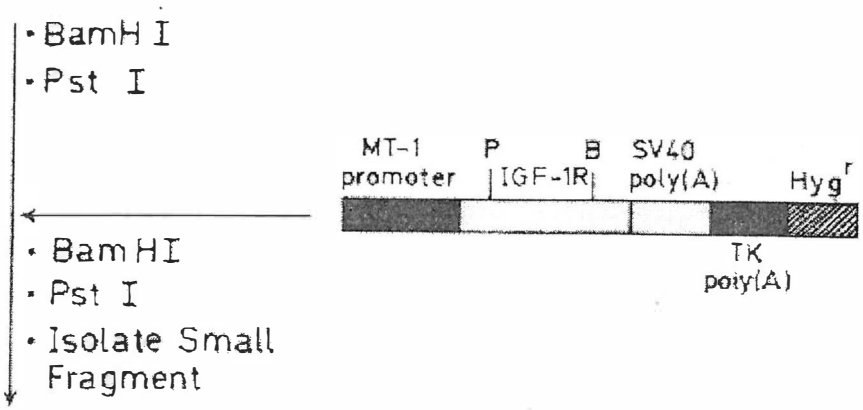
The sequence of T3 primer: (5´-AATTAACCCTCACTAAAGGG-3)

The PCR products were subjected to direct DNA sequencing using the Applied Biosystems DNA sequencer. The sequences that were obtained by using T3 or T7 primers were analyzed with BLAST program.

Transfection

Transfection of PC-3 human prostatic cancer cells was accomplished using lipofectin reagent (GIBCO/BRL, Paisley, Scotland) according to the supplier's instructions with vectors containing the IGF-IR cDNA in the antisense





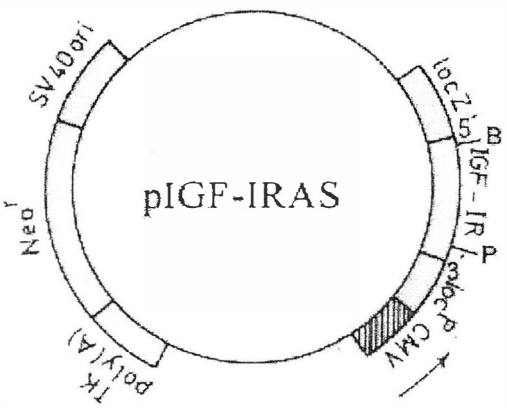


Fig. 1. Schematic representation of plasmid pIGF-IRAS. The 696-bp-long of the human IGF-IR cDNA was cloned into the pBK-CMV vector in the antisense orientation containing a constitutive CMV promoter. SV 40, simian virus 40; CMV, cytomegalovirus promoter; Neo^r, neomycin resistance gene; T3, T3 promoter; T7, T7 promoter; Tk polyA, thymidine kinase poly(A); B, BamHI; P, PstI; lacZ, lacZ gene; lac^p, lac promoter; Hyg^r, hygromycin resistance gene; MT-1, metallothionein promoter; MCS, multicloning site.

direction (pIGF-IRAS) or control vectors without an insert.

G418 (GIBCO/BRL, Paisley, Scotland) was then added at the optimal concentration. For determining the

optimal concentration of G418, the wild-type PC-3 cells (1.9×10^4) were plated on each well of 24-well plates in RPMI-1640 medium supplemented with 10% FBS and different concentrations of G418 (0, 0.2, 0.4, 0.6, 0.8 mg/mL).[25] Every two days, the medium was replaced by fresh medium and the cells from triplicate wells were counted (as mentioned above). The stable transfectants that survived after the G418 selection were further expanded under continued G418 selection.[25]

Iodination of hIGF-I

Human IGF-I was iodinated by the chloramine-T method.[26] A typical reaction mixture contained 3 μg hIGF-I (Roche Molecular Biochemicals, Mannheim, Germany), $Na^{125}I$ (300 μCi, Amersham), and 3μg chloramine-T in 0.05 M phosphate buffer, pH 7.4. The reaction was stopped after 2 min by the addition of 3 g (sodium metabisulfite. Free iodine was removed by gel filtration (Sephadex S-100). The column was equilibrated and eluted with 0.05 M phosphate buffer, pH 7.4, containing 0.1% BSA. Specific activity that was calculated using the recovery of radiolable-protein fraction, was 70.7 μCi / μg.

^{125}I-IGF-I binding assay

Cells suspended in RPMI-1640 medium supplemented with 10% FBS were plated at a cell density of 1×10^5 on each well of 24-well plates (NUNC, Roskilde, Denmark) and incubated for 2 days. Wells were then rinsed with ice-cold PBS and preincubated for 30 min at 25°C with serum-free binding buffer (SF-BB), consisting of RPMI-1640, 25 mM HEPES, and 0.1% fatty acid-free BSA. Supernatant was replaced with 0.3 ml of SF-BB containing various concentrations of radiolabeled IGF-I (0.2-6.4 nM) in triplicate. To determine nonspecific binding, incubations were performed in the presence or absence of unlabeled IGF-I (0.22 μM). Following incubation for 5 h at 25°C, medium was removed and wells were washed 3 times with ice-cold PBS. Finally, cells were extracted in 1 N NaOH and the amount of cell-bound label was measured in a gama counter (WALLAC, 1470 WIZARD™ Automatic Gamma Counter, Finland). Specific binding (SB) was determined by subtracting nonspecific binding (NSB) from total binding (TB). According to the method of Scatchard,[27] the Bound/Free of ^{125}I-IGF-I was plotted as a function of IGF-I bound to the cells. Scatchard equation is:

$B/F = -(1/kd)\times B + n/kd$

B = Ligand bound to the cells

F = Free ligand

n = The number of binding sites for ligand

Kd = dissociation constant

RESULTS

The construction of the pIGF-IRAS that was used for transfection of PC-3 cells is shown in Fig. 1. The presence of the IGF-IR cDNA insert in the antisense direction was confirmed by sequencing of the vector using the T7 or T3 primer. Fig. 2a shows the PCR product using T7 and T3 primers, the IGF-IR cDNA insert, pBK-CMV vector digested with BamHI/PstI and pIGF-IRAS. The sequences that obtained from sequencing the vector were analyzed with BLAST program. Fig. 2b shows the sequence (using T7 primer) of insert that is started from the nucleotide position 42 in exon 1 to exon 3 of IGF-IR cDNA. The optimal concentration of G418 for PC-3 cells was 0.4 mg/mL (Fig. 3). The transfected cells were grown in the presence of G418 (0.4 mg/mL) for 2-3 weeks following transfection. The successful transfectants were maintained in culture for further growth.

Radioligand-binding assay was performed in order to characterize the number of IGF-I receptors on antisense- and control-transfected PC-3 cells. The data

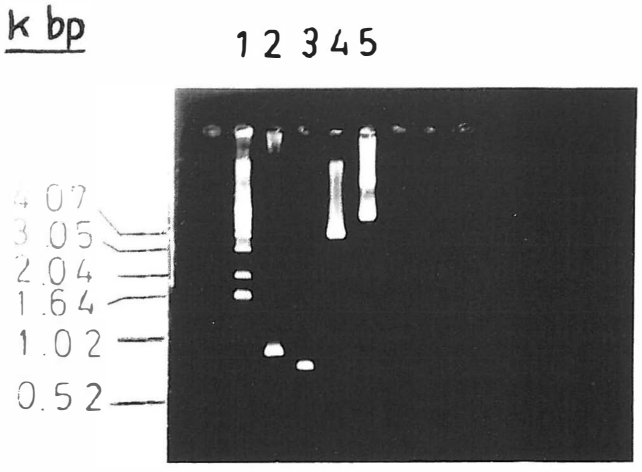


Fig. 2. (a): Lane 1, 1 Kb DNA ladder size marker; Lane 2, The PCR product using T7 and T3 primers; Lane 3, the IGF-IR cDNA insert; Lane 4, The pBK-CMV vector digested with BamHI/PstI; Lane 5, pIGF-IRAS.

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emb|X04434                                                    |HSIGFI
          Length = 4989

 Score = 1007 bits (508), Expect = 0.0
 Identities = 626/654 (95%), Gaps = 10/654 (1%)
 Strand = Plus / Plus

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2b

Fig. 2. (b): The BLAST search of sequence the pIGF-IRAS vector that obtained using the T7 primer. The sequence of insert (Query) is started from the nucleotide position 42 in exon 1 of human insulin-like growth factor receptor (Sbjct) and continued to exon 3 of IGF-IR cDNA.

in Figure 4 shows the saturation curve of the binding of ^{125}I-IGF-I to the control- and antisense-transfected PC-3 cells. Scatchard analysis of data yielded a linear plot, that suggested these cells express a single class of IGF-I binding sites. The number of IGF-I binding sites and dissociation constant was calculated from the Scatchard equation. The expressed receptor on the antisense transfectants has same affinity, as compared to that of control cells. The kd for antisense- and control- transfected PC-3 cells are 2.98 and 2.91 nM, respectively.

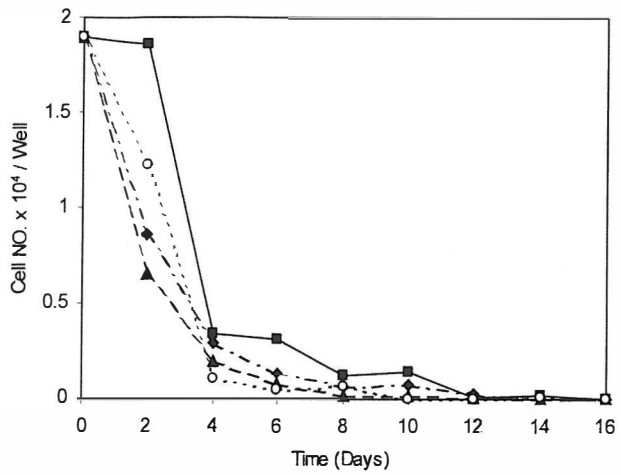

Fig. 3. The dose response curve of PC-3 cells to G418. 0.2 mg/mL (■), 0.4 mg/mL (●), 0.6 mg/mL (▲), 0.8 mg/mL (○) of G418.

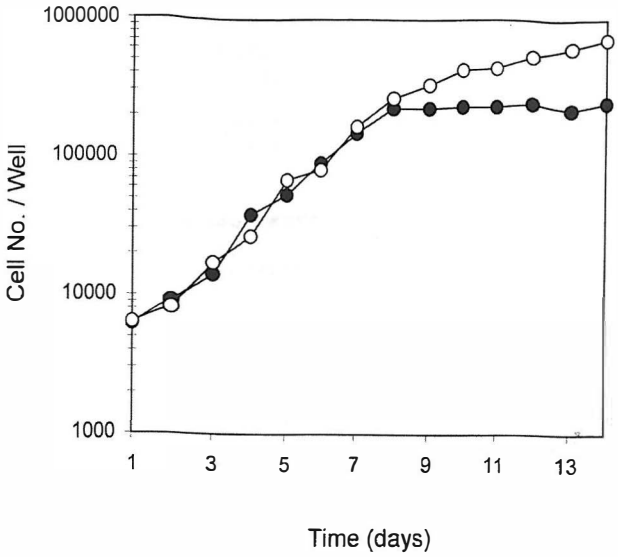

Fig. 5. Growth curve for the control- (○) and antisense- (●) transfected PC-3 cells. The data are the means of triplicate determinations.

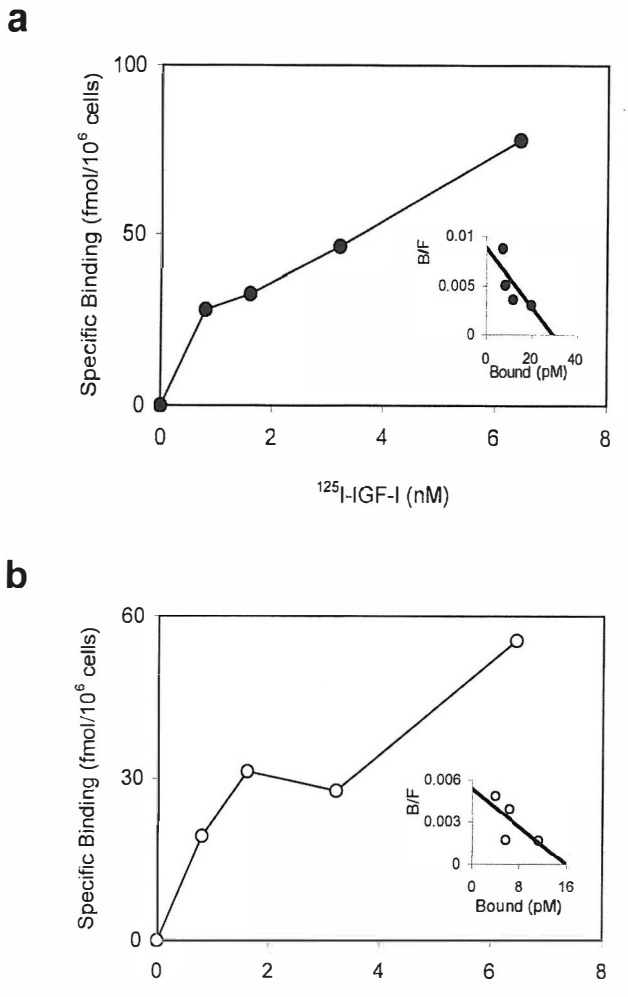

Fig. 4. Saturation of 125I-IGF-I receptor in the control- (a) and antisense- (b) transfected PC-3 cells. The Bound/Free of 125I-IGF-I is plotted as a function of hIGF-I bound, according to the method of Scatchard.[27] To determine nonspecific binding, incubations were performed in the presence or absence of unlabeled IGF-I (0.22 μM).

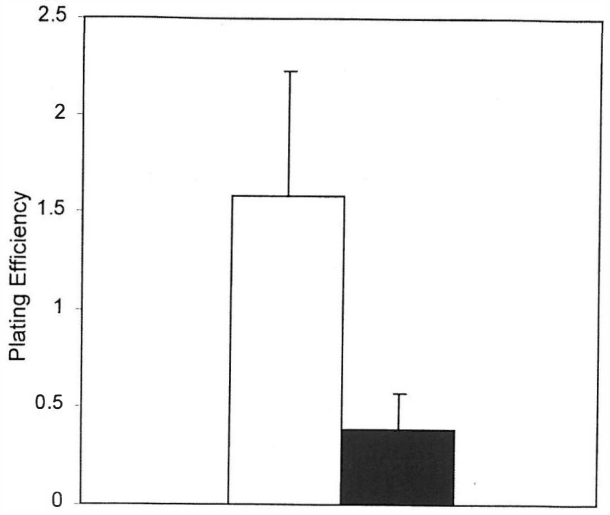

Fig. 6. The plating efficiency (PE) of the antisense- (■) and control- (□)transfected PC-3 cells cultured in agar medium. The data are the means of triplicate determinations (mean ± standard deviation).

The number of receptors on the control-transfected cells was 4.82×10^4 receptors/cell. The antisense-transfected cells express 2.88×10^4 receptors/cell. This shows a 40.2% reduction in the number of IGF-IR on the antisense-transfected PC-3 cells relative to the control-transfected PC-3 cells.

Transfection of PC-3 cells with pIGF-IRAS vectors did not alter the growth properties of these cell lines in monolayer cultures significantly (Fig. 5). The popula-

tion doubling time for control- and antisense-transfected PC-3 cells as calculated from growth curves was 35.15h and 35.16h, respectively.

Transfection of PC-3 cells with the antisense containing vector resulted in the reduction in the transformed phenotype of these cells as revealed by the decrease in the plating efficiency of these cells. Fig. 6 shows the PE of control- (1.58%) and antisense- (0.39%) transfected PC-3 cells.

DISCUSSION

The insulin-like growth factor I receptor (IGF-IR) plays an essential role in the establishment and maintenance of transformed phenotype. Suppressing the function or expression of IGF-IR by antibodies or in dominant negative mutants of the IGF-IR can reverse the transformed phenotype in many rodent and human tumor cell lines.[28-30]

Antisense RNA approaches for cancer therapy are being developed that aim to intervene at various steps in growth factor receptor-signaling pathways. One of the interesting examples has been the IGF-I receptor. For various cell types, reduction in the level of IGF-IR by IGF-IR antisense RNA strategies has markedly reduced the *in vitro* anchorage-independent colony formation in soft agar and *in-vivo* tumorgenicity.[17,31-32]

In the present study, we have stably transfected PC-3 prostate cancer cells with an IGF-IR antisense expression plasmid. This vector contained 696 bp of the human IGF-IR cDNA in the antisense orientation relative to CMV promoter. The stable transfected prostate cancer cells have maintained the reduced level of IGF-IR for up to one year. In a study, the rat PA-III prostate cancer cell line was transfected with an episome-based vector containing the IGF-IR cDNA in the antisense direction relative to CMV promoter. The PA-III transfected cells lost their ability to produce antisense RNA to IGF-IR after 10 days in the selective medium.[33] No previous work has reported the long-term establishment of transfected prostate cancer cells that express IGF-IR antisense RNA with CMV promoter.

Our quantitative data indicated that the antisense strategy adapted here was able to reduce the number of IGF-I receptor molecules by 40.2% in the antisense-transfected PC-3 prostate cancer cell line. The reduction in the PE of PC-3 antisense-transfected cells was 75.4% relative to control-transfected cells. It seems that there was a good correlation between the level of IGF-IR and inhibition of transformation in soft agar.

The fact that transfection of prostate cancer cell lines with antisense vectors against IGF-IR did not alter the growth of these cells in monolayer cultures indicate that cell proliferation (manifested by cell growth) and transformed phenotype (manifested by colony formation in soft agar) are controlled by different pathways. This phenomenon has been observed in other cases such as melanoma cell lines.[18]

These results confirm that the IGF-IR has a more important role in the maintenance of the transformed phenotype than in growth of cells and antisense strategies reverse the transformed phenotype in human androgen-independent prostate cancer cell line, PC-3. The results of this study could provide a basis for designing antisense strategies for treatment of prostate cancer that would involve targeting the IGF-IR at the cellular level by preventing expression of IGF-IR in prostate cancer cells.

ACKNOWLEDGEMENT

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