Knockdown of IGF-I R by antisense oligodeoxynucleotide augments sensitivity of T24 bladder cancer cells to mitomycin

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KEY WORDS insulin-like growth factor I; receptors; autocrine; drug resistance; apoptosis; bladder neoplasms

ABSTRACT

AIM: To investigate whether insulin-like growth factor I receptor (IGF-1 R) was involved in drug resistance of bladder cancer cells. METHODS: RT-PCR was used to detect the mRNA expression of IGF-I, IGF-II, and IGF-I R in T24 cells and normal urothelial cells. Flow cytometry and MTT tests were used to assess the effect of antisense oligodeoxynucleotide (ODN) on drug sensitivities and apoptosis of T24 cells to mitomycin (MMC). Western blot was used to analyze the effect of ODN on expression of IGF-1 R protein. RESULTS: mRNA of IGF-I, IGF-II, and IGF-1 R were strongly expressed in serum-free cultured T24 cell line, whereas normal urothelial cells did not express these factors/receptors or only in trace levels; knockdown of IGF-1 R by antisense ODN greatly inhibited the growth of bladder cancer cells and enhanced sensitivity and apoptosis of T24 cells to MMC. CONCLUSION: These results suggested that blockage of IGF-1 R signaling might potentially contribute to the treatment of bladder cancer cells which were insensitive to chemotherapy.

INTRODUCTION

Transitional cell carcinoma (TCC) of the bladder represents the fifth most prevalent malignancy in Western population. Bladder instillation represents the most significant progress in the management of superficial bladder cancer over the past twenty years. But a major problem in the management of bladder cancer is the low sensitivity to chemotherapy and the high recurrence of bladder cancer after transurethral resection, which occupies a large proportion (approximately 40%) among bladder cancer patients. So drug resistance remains a major and difficult problem to resolve in bladder cancer chemotherapy. This phenomenon has often been ascribed to some mechanisms, such as the overexpression of multidrug transporters P-glycoprotein, multidrug resistance related protein (MRP), and other variables closely implicated DNA repair and induction/modulation of apoptosis, such as P53 and the Bcl-protein family. Furthermore, it has been recently shown that certain growth factors (insulin-like growth factors, IGF, etc.) might be involved in the mechanism of drug resistance. Clearly, these findings suggest the design of new strategies might improve bladder cancer response to chemotherapy.

The insulin-like growth factor system is widely involved in human carcinogenesis. The type I insulin-like growth factor receptor (IGF-I R) is a transmembrane protein tyrosine kinase which mediated the biological effects of IGF-I and most of the actions of IGF-II. It plays an important role in both normal and abnormal growth, particularly in anchorage independent growth. Impairment of its function caused apoptosis of some kinds of cancer cells and inhibition of tumor growth in experimental animal. Some investigators also provided evidences that IGF-I R signaling might be implicated in the mechanism of drug resistance in human breast cancer. IGF-I R activation could afford drug protection either by affecting the negative regulators of the apoptosis pathway, Bel-2 and Bel-xl, or by altering activity of the ced-3/ICE-like proteases. P33, the Bcl-protein family, is now confirmed and prominent in the mechanism of drug resistance. More recently, Luo et al demonstrated that IGF-I R system protected colon cancer cells from apoptosis induced by cytotoxic agents, which acquired drug
resistance might be mediated by multiple mechanisms, including promoting expression of multidrug resistance gene (mdr-1 gene), etc. Lowering IGFR-I R system might thus represent an attractive strategy to be pursued for chemopreventive purpose.

Bladder cancer cells were found to overexpress high levels of functional IGFR-I receptors and IGFR-II [10-16]. There were considerable evidences that the antiapoptotic function of IGFR-I R signaling contributed to progression, invasion, and metastasis of bladder cancer [20]. But the following problems remained to be further elucidated in bladder cancer research, which the main objectives of our studies were concerned about; whether IGFR-I R signaling pathway is involved in autocrine growth of human bladder cancer; whether IGFR-I R is involved in the growth stimulating activity and drug resistance of bladder cancer cells or not; can targeting against IGFR-I R gene significantly enhance drug sensitivity of urinary bladder cancer cells to mitomycin (MMC, one of the most effective drug used for the intravesical chemotherapy to bladder cancer).

MATERIALS AND METHODS

Agent Mitomycin (MMC) was obtained from Feiyun Pharmaceutical Factory (Hubei, China); 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl tetrazolium bromide (MTT) and propidium iodide (PI) were purchased from Sigma Chemical Co; TRIZol and RPMI-1640 medium were the products of Gibco. NCI H69 and K562 cell line were purchased from Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences.

Cells culture T24 cells line, established from high grade and invasive human urinary bladder cancer patient and generally considered as representation for research of drug sensitivity [21], was kindly supplied by Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were cultured in RPMI-1640 medium supplemented with 5 % heat-inactivated fetal calf serum (FCS), benzylpenicillin 100 kU/L, streptomycin 100 mg/L. NCI H69 (a small-cell lung cancer line), K562 (a human erythro-leukemia cell line) were cultured in vitro as described above.

Primary cultured normal human urethelial transitional epithelial cells, were established from normal ureters (from donors for kidney transplantation) by methods described previously [22]. Cells were cultured in PRMI-1640 medium supplemented with 10 % FCS, non-essential amino acids 10 μmol/L, dextrose 2.7 g/L, hydrocortisone 1 mg/L (Sigma, St Louis, MO), transferrin 5 mg/L, insulin 10 mg/L, epidermal growth factor 10 μg/L, streptomycin 100 mg/L, and benzylpenicillin 100 kU/L in a humidified atmosphere of 5 % CO2.

RT-PCR Total RNA was prepared from T24 cells by TRIZol. Synthesis of cDNA was carried out with reverse transcriptase (MMLV, 0.1 MU/g RNA, Gibco) from 1 μg of total RNA. Reverse-transcribed single-stranded cDNA 2 μL was subjected to PCR in 50 μL of 1 x buffer [Tris-HCl 10 mmol/L (pH 8.3); KCl 50 mmol/L; MgCl2 1 mmol/L; dNTPs 50 μmol/L; 2 units of Thermus aquaticus polymerase (Sigma); 0.2 μmol/L corresponding primers of the indexes to be detected and β-actin]. All of the primer sequences used in the present study, PCR product sizes, and PCR conditions are listed in Tab 1. Oligonucleotides specific

Tab 1. RT-PCR primer sequence, conditions, and the PCR product sizes.

<table>
<thead>
<tr>
<th>Primer sequence (product sizes)</th>
<th>PCR conditions</th>
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<tr>
<td>internal control + β-actin (211 bp)</td>
<td>same as complified IGFR-I (or IGFR-II or IGFR-I R)</td>
</tr>
<tr>
<td>F: 5'-TCTGATGACACACAGACGCTG3'</td>
<td>(94 °C 1 min, 58 °C 1 min, 72 °C 5 min, 30 cycles)</td>
</tr>
<tr>
<td>R: 5'-GACTGCTTCTGAGCTGATCC3'</td>
<td></td>
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<tr>
<td>IGFR-I (413 bp)</td>
<td></td>
</tr>
<tr>
<td>F: 5'-TCTGATGACACACAGACGCTG3'</td>
<td>(94 °C 1 min, 55 °C 1 min, 72 °C 5 min, 28 cycles)</td>
</tr>
<tr>
<td>R: 5'-GACTGCTTCTGAGCTGATCC3'</td>
<td></td>
</tr>
<tr>
<td>IGFR-II (300 bp)</td>
<td></td>
</tr>
<tr>
<td>F: 5'-TCTGATGACACACAGACGCTG3'</td>
<td>(94 °C 1 min, 55 °C 1 min, 72 °C 5 min, 35 cycles)</td>
</tr>
<tr>
<td>R: 5'-GACTGCTTCTGAGCTGATCC3'</td>
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to IGF system mRNA were designed from known genomic and cDNA information. The amplified products were separated by electrophoresis on 1.5% agarose gel and visualized by staining the gel with ethidium bromide. Each RT-PCR was repeated three times using different preparations of RNA. Internal control β-actin RT-PCR was done on all of the samples simultaneously.

**MIT assay** Exponentially growing T24 cells were seeded in 96-well, flat-bottomed plates at 1 x 10^4 cells/L. MTT (5 g/L) 20 μL was added into each well. The blue dye taken up by the cells after 4-h incubation was dissolved in MeSO (100 μL/well), and its optical density at 495 nm was read on an automated microplate reader (Bio-Tech. Winooski, VT). Results of a preliminary study with the MIT assay showed that the absorption was directly proportional to the number of cells.

**Apoptosis assay** The attached cells were treated with trypsin/edetic acid, pelleted together with floating cells, washed once with ice-cold PBS, and re-suspended in a hypotonic fluorochrome solution containing propidium iodide 50 mg/L in 0.1% sodium citrate plus 0.03% (v/v) Nonidet P-40. After 30 min of incubation, the samples were filtered through 40-μm nylon mesh cloth, and their fluorescence was analyzed as single-parameter frequency histograms using a FACScan flow cytometry (Becton Dickinson, Mountain View, CA). According to Darnyckiewicz et al., apoptosis was determined by evaluating the percentage of events accumulated in the subdiploid peak.

**Effect of antisense ODN on IGF-I R protein expression by Western blot** Antisense strategy for depleting IGF-I R was described previously. The sequence was: 5’ AAG TCC GCC GGA GGA-3’ (sense), 5’-TCC GCC GGA GGA-3’ (antisense). They represent codon 2-7 of the prepropeptide, and have been shown previously to effectively decrease the numbers of IGF-I receptors. T24 cells were seeded in 5% FCS medium to subconfluence and then incubated in medium containing antisense ODN (added at intervals of 24 h), sense ODN (added at intervals of 24 h) or MEM 0.2% SDS (treated for 1 h, then replaced by drug-free culture medium), alone or cotreatment for 72 h and harvested for analysis.

To evaluate the effects of ODN on IGF-I R protein level, T24 cells were transferred to microtubes and lysed in ice-cold lysis buffer (PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, phenylmethylsulfonyl fluoride 1 mmol/L, and aprotonin 10 μg/mL) for 20 min. The supernatant was collected after centrifugation at 10,000 x g, 4°C for 20 min. Protein concentration in the lysate was determined by the Bio-Rad Bradford protein assay kit (Herlev, CA). Protein 0.5 μg was denatured and fractionated on a 7.5% SDS polyacrylamide gel and electrophoresed onto a nitrocellulose membrane (Hybond-C, Amersham). The membrane was incubated consecutively with anti-IGF-I R monoclonal antibody H7 (Santa Cruz Biotechnology, INC. diluted 1:2000) and horseradish peroxidase-conjugated anti-rabbit IgG. The blots were visualized by enhanced chemiluminescence (ECL) (Amersham, Pharmacia Biotech).

**Statistical analysis** Statistical analysis was performed with Student's t test or Chi-square test, and P < 0.05 was considered significant.

**RESULTS**

**Autocrine expression of IGF-I, IGF-II, and IGF-I R mRNA of T24 urinary bladder cancer cells** To evaluate expression of IGF-I, IGF-II, and IGF-I R mRNA in urinary bladder cancer cells and primary normal urothelial cells, RT-PCR was performed on extracted mRNA aliquots from serum-free cultured cells. T24 bladder carcinoma cells tested strongly expressed IGF-I, IGF-II, and IGF-I R mRNA (Fig 1). In contrast, IGF-I, IGF-II, and IGF-I R mRNA were not expressed or expressed only at trace levels by

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**Fig. 1.** Analysis of IGF-I, IGF-II or IGF-I R mRNA by RT-PCR. Co-amplification of IGF-I, IGF-II or IGF-I R with β-actin. RT-PCR products were analyzed on ethidium bromide-stained agarose gels and bands were examined by scanning densitometry. NCI H69, K562 cells were used as respective positive controls. Lane N: normal urothelial cells; Lane M: marker (bp).
normal urothelial cells. Expression of β-actin was used as an internal control.

Antisense ODN effectively reduces protein synthesis of IGF- I R and enhances drug sensitivity and apoptosis of T24 cells to MMC We first examined the effect of antisense ODN targeting against IGF- I R on protein synthesis. Representative example of protein synthesis in T24 cells treated with antisense ODN was shown in Fig 2. The expression intensities of IGF- I R protein were quantified by densitometric scanning. The values represented x ± s of three different experiments performed in duplicate. The values of protein expression were as following: 1614 ± 156 (antisense-untreated T24 group), 621 ± 27 (antisense ODN 5 μmol/L treated T24 cells), 276 ± 186 (antisense ODN 10 μmol/L treated T24 cells). IGF- I R protein was downregulated by 61.3 % ( P < 0.01, antisense ODN 5 μmol/L treated T24 cells), 77 % ( P < 0.01, antisense ODN 10 μmol/L treated T24 cells). Data showed antisense ODN could effectively reduce IGF- I R protein expression.

Fig 2. Analysis of IGF- I R protein level of T24 cells treated with antisense ODN for 72 h by Western blot. Lane 1: positive control ( K562 cells); Lane 2: untreated T24 cells; Lane 3, 4, 5: sense ODN (2 μmol/ L, 5 μmol/L, and 10 μmol/L); Lane 6, 7, 8: antisense ODN (2 μmol/L, 5 μmol/L, and 10 μmol/L).

We then examined the effect of antisense ODN on drug sensitivity of T24 to MMC. As shown in Fig 3, treatment of T24 with IGF- I R antisense ODN for 72 h enhanced the sensitivity of bladder carcinoma cells to MMC 0.2 μmol/L. Apoptotic analysis by flowcytometry also confirmed that blocking IGF- I R signaling by antisense ODN could cause targeted cells to be more sensitive to MMC-induced apoptosis (Fig 4).

DISCUSSION

The present study yielded the following: The mRNAs of IGF- I , IGF- II , and IGF- I R were strongly expressed in serum-free cultured T24 bladder cancer cells, whereas normal urothelial cells did not express these factors/receptors or only in trace levels; antisense ODN could effectively reduce protein synthesis of IGF- I R and significantly enhance apoptotic sensitivity of T24 cells to MMC. Our observations indicated a possible mechanism by which bladder cancer cells acquired an additional growth advantage over their benign counterparts; namely, by acquisition of a better IGF-producing capacity, and also suggested that IGF- I R signaling pathway may serve as potential candidates for clinical intervention in the sensitization of drug-resistant bladder carcinoma cells to cytotoxic drugs.
MMC is the first-line intravesical chemotherapeutic agent for superficial bladder cancer. The mechanism of action of MMC is largely attributable to its cross-linking with DNA, particularly at guanine/ cytosine-rich regions. The drug is most effective when given in the late G1 and early S phases of the cell cycle.

Here, we evaluated the potential therapeutic application of antisense ODN targeted against IGF-1 R gene on urinary bladder cancer cells. It should be pointed out: (1) Targeting the IGF-1 R is more efficient than targeting its ligands because targeting one ligand leaves other one free to activate the IGF-1 R [4]. (2) The bladder is particularly amenable to such experimental treatment, because its special anatomic structure and localized therapy is possible by intravesical administration, thus minimizing side effects [20]. (3) It has been shown that antisense ODN against IGF-1 R gene is remarkably non-toxic in vivo [7]. In this study, synergy with antisense ODN was achieved with very low concentrations of MMC agent, far below under the clinical therapeutic dose, thus minimizing their toxicity and maximizing their therapeutic application.

In summary, we concluded that acquisition of IGF-producing capability may give a growth advantage over non-neoplastic urethelial cells. Further antisense blockage of IGF-1 R signaling could augment bladder cancer cells sensitivity to MMC, indicating potential clinical application of IGF-1 R antisense ODN for bladder cancers. Nevertheless, these various approaches await appropriate pre-clinical in vivo studies to delineate their effect and toxicity.

REFERENCES

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反义脱氧寡核苷酸阻断 IGF-1 R 通路可增强 T24 膀胱癌细胞对丝裂霉素的敏感性

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关键词：胰岛素样生长因子 I；受体；自分泌；抗药性；细胞凋亡；膀胱癌

目的：探讨 IGF-1 R 信号旁路是否参与膀胱癌细胞的耐药性。方法：RT-PCR 法检测 T24 膀胱癌细胞和正常尿路上皮细胞 IGF-1、IGF-2 及 IGF-1 R mRNA 表达。流式细胞技术和 MTT 试验检测反义脱氧寡核苷酸能否增强 T24 细胞对丝裂霉素药物敏感性及凋亡易感性；免疫印迹技术检测反义脱氧寡核苷酸对 T24 膀胱癌细胞 IGF-1 R 蛋白表达的影响。结果：IGF-1、IGF-2 及 IGF-1 R mRNA 在无血清培养的 T24 细胞中表达，而正常尿路上皮细胞不表达或仅微量表达这些生长因子或受体；反义脱氧寡核苷酸阻断 IGF-1 R 通路可以显著抑制膀胱癌细胞的生长并可增强 T24 细胞对丝裂霉素的敏感性及凋亡易感性。结论：阻断 IGF-1 R 信号通路可有望治疗对化疗不敏感的膀胱癌。