Interleukin-24 Transduction Modulates Human Prostate Cancer Malignancy Mediated by Regulation of Anchorage Dependence

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Abstract. *Background: Hormone therapy and chemotherapy are not effective for castrate-resistant prostate cancer, thus development of novel treatment strategies is required. Gene therapy involving transient high-copy transfection of interleukin (IL)-24 with an adenoviral vector can exert antitumor activity; however, the effects of stable IL-24 transfection are not fully understood. The aim of this study was to investigate the effects of IL-24 overexpression in prostate cancer cells, in vitro. Materials and Methods: DU145 cells were transfected the IL-24 gene using a retroviral vector. Apoptosis induction was investigated by the cell death detection ELISA, and the gene expression was analyzed by real time RT-PCR. Results: IL-24 transduction suppressed the growth of prostate cancer and induced tumor cell apoptosis. In addition, up-regulation of epithelial markers and down-regulation of mesenchymal markers were noted, suggesting that tumor aggressiveness was reduced. Conclusion: Introduction of IL-24 displays antitumor activity both by induction of apoptosis and regulation of anchorage dependence.*

Prostate cancer has the highest cancer morbidity rate and is the second most frequent cause of cancer-related death in the USA, being one of the most frequent malignancies among men (1). Prostate cancer often progresses silently and may cause metastases, especially in osseous sites (2). Early detection of prostate cancer by measurement of prostate specific antigen has led to a rapid increase of patients with this tumor type (3, 4).

Since it is difficult to effectively control metastatic castrate-resistant prostate cancer with hormone therapy or chemotherapy (5, 6), development of new treatments is

required. Interleukin-24 (IL-24), member of the IL-10 family, is a monomeric glycoprotein with a molecular weight ranging from 35 to 40 kDa that is mainly produced by splenocytes and peripheral blood leukocytes (7-9). The IL-24 receptor has two heteroforms, IL-20RA/IL-20RB and IL-22RA/IL-20RB (10), and binding of IL-24 to these receptors activates the JAK/STAT signaling pathway in normal cells (11).

IL-24 (also known as MDA-7) was initially discovered as an antitumor factor that suppressed the growth of melanoma cells and promoted their terminal differentiation after treatment with interferon and mezerein (12, 13). It was reported that IL-24 gene expression in cancer cells shows a negative correlation with tumor invasion and metastasis (14, 15). Introduction of the IL-24 gene using an adenoviral vector suppresses the growth of several types of tumor cells, while having no such effect on normal cells (16, 17). According to previous studies, transient adenovirus-based transfection of cancer cells with IL-24 shows antitumor activity by induction of endoplasmic reticulum stress and activation of caspases; however, the effect of constitutive IL-24 expression is unknown.

In this study, we established a prostate cancer cell line with stable overexpression of IL-24 by using a retroviral vector. The growth inhibitory effect of IL-24 was investigated by evaluation of cell viability, BrdU incorporation, and apoptosis. We also observed other antitumor effects after retroviral introduction of IL-24, such as regulation of anchorage dependence.

Materials and Methods

Cells and reagents. The human prostate cancer cell line DU145 (#RCB2143; Riken BioResource Center, Tsukuba, Japan) was cultured at 37°C in RPMI 1640 medium (Sigma Aldrich, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Gibco by Thermo Fisher Scientific, Waltham, NY, USA) and 100 units/ml of penicillin plus 10 µg/ml of streptomycin (Gibco, Tokyo, Japan) under a humidified atmosphere of 95% air and 5% CO₂.

A packaging cell line (PT67) was purchased from Clontech (via Takara Bio Inc., Tokyo, Japan) and NIH/3T3 cells were kindly provided

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by Dr. Majima (Kitasato University School of Medicine), with both cell lines being cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% FBS and 100 units/ml of penicillin plus 10 µg/ml of streptomycin under a humidified atmosphere of 95% air and 5% CO₂.

Recombinant human IL-24 (rhIL-24) was purchased from R&D Systems (Minneapolis, MN, USA).

Introduction of the IL-24 gene. Human IL-24 cDNA was synthesized by Medical & Biological Laboratories (BML, Aichi, Japan) and a recombinant plasmid was designed (pSMARTIDT-hIL-24). After pSMARTIDT-hIL-24 was digested with Not I and Cla I, the resulting fragments were cloned into the Not I/Cla I sites of the pBlueScript SK II amplification vector (pBluescript-hIL-24). Next, the Not I/Cla I fragments were cloned into the pDON-5 Neo deficient retroviral vector to obtain pDON-hIL-24, which was subsequently transfected into PT67 packaging cells, followed by selection with G418 to obtain PT67-hIL-24 cells. PT67 packaging cells only transfected with pDON5-Neo were used as a control (PT67-EV cells). The IL-24 sequence cloned into pDON-5 neo was determined by DNA sequencing using an ABI 3100 genetic analyzer (Applied Biosystems by Thermo Fisher Scientific), and was verified by comparison with a database (accession no. BC009681).

To evaluate the infectious titer of recombinant retrovirus containing the hIL-24 gene in the culture supernatant of PT67-hIL-24 and PT67-EV cells, supernatant diluted by 10⁻¹ to 10⁻⁵ was used to infect 5×10⁴ NIH/3T3 fibroblasts, followed by selection with G418 antibiotic. The retroviral vector was also used to infect LLC cells, followed by selection with G418. The resulting cell lines were designated as DU145-hIL-24 and DU145-EV, respectively.

RT-PCR and real time RT-PCR. Genomic DNA-free total RNA was extracted and purified from cells or tumor tissues by using the RNeasy Plus Mini Kit (Qiagen, Venlo, Netherlands) and the TURBO DNA-free Kit (Ambion by Thermo Fisher Scientific). Then 2 µg of total RNA was used for synthesis of cDNA with Omniscript RT Kit (Qiagen) in a total volume of 20 µl.

A 1 µl aliquot of cDNA was submitted for PCR in a volume of 50 µl. PCR involved activation at 94°C for 15 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min using a thermal cycler (GeneAmpPCR System 9700: Applied Biosystems) and SYBR Premix Dimer Eraser (Takara Bio Inc.). The oligonucleotide primers are listed in Table I.

ELISA. DU145-hIL-24 cells or DU145-EV cells were seeded at a density of 5×10⁴ cells/well in 6-well culture plates (AGC Techno Glass, Shizuoka, Japan) and then incubated for 24 h. After medium exchange, culture was continued for another 48 h and the supernatant was collected by centrifugation at 1,500 rpm for 5 min. The IL-24 level in the supernatant was analyzed by using a Human IL-24 DuoSet ELISA kit (R&D Systems).

Cell proliferation assay. DU145-hIL-24 cells or DU145-EV cells were seeded at a density of 1×10³ cells/well in 96-well culture plates (AGC Techno Glass) and then incubated for 24 h. After medium exchange, culture was continued for another 96 h, and then cell proliferation was investigated by the WST assay (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan) and a BrdU ELISA Kit (Roche, Mannheim, Germany).

Phalloidin staining. DU145-hIL-24 cells or DU145-EV cells were seeded at a density of 5×10³ cells on 13 mm diameter poly-L-lysine-coated cover slips (Matsunami Glass Ind., Osaka, Japan) placed at the bottom of a 24-well culture plate (AGC Techno Glass), and then were incubated for 24 h. After culture for an additional 96 h, cells were fixed in 4% PFA, rinsed three times in PBS, permeabilized with 0.1% Triton-X in PBS, and rinsed three times in PBS. Subsequently, the cells were stained with phalloidin conjugate solution (Abcam, Cambridge, UK) at room temperature for 90 min, and then were rinsed four times in PBS. Finally, the cells were mounted by using Vectashield HardSet Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA), and were observed under a type BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

Detection of apoptosis. DU145-hIL-24 cells or DU145-EV cells were seeded at a density of 1.0×10³ cells/well in 96-well culture plates, and then incubated for 24 h. After culture for a further 96 h, the cells were lysed and apoptosis was investigated by a cell death detection ELISA Plus Kit (Roche, Mannheim, Germany) after removal of the culture medium.

Statistical analysis. All experiments were conducted three times. Results are expressed as the mean±standard deviation (SD). Comparisons between two groups were performed using Student's *t*-test and *p*<0.05 was considered to indicate statistical significance.

Results

Characterization of DU145-hIL-24 cells. The process of constructing pDON-5 Neo-hIL-24 is summarized in Figure 1A. A defective retrovirus vector was assembled using pDON-5 Neo-hIL-24 and was employed to infect DU145 cells at a MOI of 10⁻⁴ to obtain DU145-hIL-24 cells. IL-24 mRNA (429 bp) was clearly expressed in DU-145-hIL-24 cells, but was undetectable in control DU145-EV cells. On the other hand, expression of the neomycin resistance gene derived from the retroviral vector (Neo) and expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were clearly seen in both cell lines (Figure 1B). In culture supernatant of DU145-IL-24 cells, the IL-24 protein concentration was 3.8 ng/ml, but IL-24 was undetectable in the supernatant of DU145-EV cells (Figure 1C).

Induction of apoptosis in DU145-hIL-24 cells. Proliferation of DU145-hIL-24 and DU145-EV cells was evaluated by the WST assay and by ELISA for BrdU. At 96 h after cell seeding, proliferation of DU145-IL-24 cells was about 30% lower compared to DU145-EV cells, according to the WST assay and BrdU ELISA (Figure 2A and B).

A cell death detection ELISA was also performed to determine whether the suppressive effect of IL-24 on cell proliferation was associated with apoptosis. This showed that apoptosis was elevated by approximately 50% in DU145-hIL-24 cells compared with DU145-EV cells (Figure 2C).

Table I. *Primer sequences.*

Target gene	Product length	Sequence (5'-3')
GAPDH	190 (bp)	F: TGGCTACAGCAACAGGGTGGTG R: TTCAAGGGGTCTACATGGCAACTG
IL-24	429 (bp)	F: GACTTTAGCCAGCAGACCCTT R: GGTTGCAGTTGTGACACGAT
Neo	515 (bp)	F: GGGCGCCCGGTTCTTTTGT R: ACACCCAGCCGCCACAGTCG
IL-20RA	682 (bp)	F: CCAGCTTCGACCAGGATTCA R: ATGCGACCAACTTACCACCA
IL-22RA	481 (bp)	F: CTGTCCGAGATCACCTACTTAGG R: GCACATTGGGGTCAGATGTTCTGTG
IL-20RB	480 (bp)	F: CAGGTGAAGCCGAGAACCTG R: CAAGCCACCCAGTGTGTAGA
E-cadherin	103 (bp)	F: TGCACCAACCCTCATGAGTG R: GTCAGTATCAGCCGCTTTCAG
Fibronectin 1	97 (bp)	F: TCGCCATCAGTAGAAGGTAGCA R: TGTTATACTGAACACCAGGTTGCA
α -SMA	88 (bp)	F: CCGACCGAATGCAGAAGGA R: ACAGAGTATTTGCGCTCCGGA
smad 7	82 (bp)	F: GCCGACTCTGCGAACTAGAGT R: GGACAGTCTGCAGTTGGTTTGA

F, Forward; R, reverse.

Apoptotic effect of IL-24 not mediated via the IL-24 receptor. To investigate whether the antitumor effect of IL-24 was mediated via IL-24 receptor signaling, tumor cells were exposed to endogenous rhIL-24. It was found that endogenous rhIL-24 had no effect on cell proliferation, even at a high concentration (10 ng/ml) (Figure 3A). In addition, expression of IL-24 heteroreceptors (IL-20RA, IL-22RA, and IL-20RB) was not found in DU145 cells, but was detected in PC-3 control cells (Figure 3B). These results imply that the antitumor effect of introduced IL-24 was independent of IL-24 receptor signaling.

Morphological changes of DU145-hIL-24 cells. Microscopy showed that DU145-hIL-24 cells grew more densely in comparison with DU145-EV cells. Fluorescent staining of actin filaments was performed to evaluate motility, and stress fibers were detected in DU145-EV cells, but not in DU145-hIL-24 cells (Figure 4A). To investigate this difference of actin filament formation, quantitative analysis of epithelial and mesenchymal markers was performed by real time RT-PCR. This revealed that expression of the mesenchymal markers fibronectin 1 and alpha-smooth muscle actin (α -SMA) was significantly decreased in DU145-IL-24 cells compared to DU145-EV cells (Figure 4B). These results indicated that introduction of IL-24 influenced the regulation of anchorage dependence.

Figure 1. Transfection of the human interleukin-24 (hIL-24) gene and its expression in prostate cancer cells. The human IL-24 gene was introduced by using a defective retroviral vector that integrated into genomic DNA and stably expressed the target gene. The hIL-24 gene was cloned into the NotI to ClaI site of the pDON-5 neo retroviral vector (designated as pDON-5 Neo-hIL-24) (A). DU145 cells were infected with pDON-5 neo-IL-24, followed by G418 selection to obtain DU145-hIL-24 cells. DU145 cells were also infected with the pDON-5 Neo empty vector to obtain control DU145-EV cells. RT-PCR was performed to assess mRNA expression by DU145-IL-24 and DU145-EV cells (B). Production of hIL-24 protein into culture supernatant after incubation for 48 h was detected by ELISA. N.D., Not detected (C). Data are the mean $\pm$ SD. ** p <0.01 (compared to DU145-EV cells).

Figure 2. Antitumor effect of interleukin-24 (IL-24) in DU145-hIL-24 prostate cancer cells. The cells were cultured for 96 h before performing growth and apoptosis assays. Tumor cell viability (A) and growth (B) were assessed by the WST assay and BrdU ELISA, respectively. Apoptosis was analyzed with cell death detection ELISA (C). Data are the mean $\pm$ SD and a representative result of three experiments is shown. * p <0.05 (compared to DU145-EV cells).

Discussion

In this study, we investigated the antitumor effect of retroviral transduction of IL-24 in prostate cancer cells, and we demonstrated that this led to inhibition of cell growth and viability along with induction of apoptosis. In addition, motility of the cancer cells was affected, with reduction of stress fiber formation and downregulation of fibronectin expression. Interestingly, the cancer cells did not express IL-

Figure 3. Effect of recombinant human interleukin-24 (hIL-24) and IL-24 receptor (IL-20RA, IL-22RA, and IL-20RB) expression by tumor cells. DU145 cells were cultured in medium containing rIL-24 or PBS control for 96 h, after which growth was assessed by the WST-8 assay (A). IL-24 receptor expression was detected by RT-PCR (B). Data are the mean $\pm$ SD and a representative result of three experiments is shown.

24 receptors and were insensitive to recombinant IL-24. These results suggest that introduction of IL-24 into cancer cells displays antitumor activity by both induction of apoptosis and regulation of anchorage dependence *via* intracellular signaling.

Thus, we found that transduction of IL-24 *via* a retroviral vector showed antitumor activity. Further investigation indicated that BrdU incorporation during cell division and induction of apoptosis occurred simultaneously, implying that suppression of growth depended on enhancement of cell

Figure 4. Morphological changes of DU145 cells after transduction of hIL-24. Observation of DU145-hIL-24 and DU145-EV cells in bright field (upper). Actin filament staining by fluorescent phalloidin (green), with counterstaining of cell nucleus by DAPI (blue) (lower) (A). Epithelial marker and mesenchymal marker expression was determined by real-time RT-PCR (B). Scale bars 50 μ m. Arrows show actin filament formation. Data are the mean $\pm$ SD of three experiments. * p <0.05 (compared to DU145-EV cells).

death *via* apoptosis rather than inhibition of mitosis. It was reported that transduction of IL-24 into human melanoma cells using an adenoviral vector induced growth suppression and apoptosis ranging between –60 and >80% *versus* control cells (17). This report indicates a difference between the antitumor effect of retrovirus vector-mediated and adenovirus vector-mediated transfection of IL-24. Kreis et al. concluded that the antitumor effect of IL-24 is mediated by a specific pathway after transfection with an adenoviral vector, and the same effect cannot be obtained by only transduction of IL-24 DNA (18). Hence, the effect of introducing IL-24 without an adenoviral vector might be limited, suggesting that it would be less efficient to only induce IL-24 DNA or plasmid-mediated gene expression, and that strong induction of apoptosis requires another cooperative factor.

In the present study, we observed a decrease of fibronectin 1 and stress fibers in DU145 cells transfected with IL-24 using a retroviral vector. In addition, actin fiber formation was decreased by introduction of IL-24, indicating that tumor cell motility was probably suppressed. Moreover, mRNA analysis showed mesenchymal marker down-regulation combined with epithelial marker up-regulation. Thus, introduction of IL-24 had the effect of suppressing cell motility and altering anchorage dependence. In the process of metastasis, tumor cells acquire the ability to cross the basement membrane and cleave stromal tissue (19), suggesting that regulation of anchorage independence by IL-24 could decrease tumor malignancy.

Our data showed that DU145-IL-24 cells secreted IL-24 protein into the culture medium. Since cytokines are generally secreted proteins and act *via* specific receptors, we examined the effect of adding rhIL-24 to cell cultures. Contrary to our expectation, rhIL-24 did not exert an antitumor effect and DU145 cells did not show strong expression of IL-24 receptors. Accordingly, it seems that IL-24 transduced by our retroviral vector acted *via* an intracellular pathway, rather than *via* an extracellular pathway. The primary sequence of human IL-24 differs from homologous cytokines, including IL-10 and the IL-19 subfamily, because it has three consensus N-linked glycosylation sites and the potential to form a single disulfide bond (20). It was reported that IL-24 is modified by glycosylation, S-S bridging, and phosphorylation, with glycosylation and S-S bridging being essential for secretion and activation of IL-24 protein (21). Panneerselvam et al. investigated the phosphorylation of IL-24, and reported that phosphorylated IL-24 suppressed the oncogene pAkt and promoted mTOR-mediated induction of apoptosis (22). Also, intracellular IL-24 protein was reported to interact directly with protein kinase RNA-activated, a double-stranded RNA-activated protein kinase (23). However, the mechanisms involved in

phosphorylation of IL-24 protein and the subsequent intracellular signaling pathways are largely unknown and further investigation is required.

In conclusion, we showed that retrovirus-mediated constitutive overexpression of IL-24 suppressed prostate cancer cell proliferation and attenuated malignancy *via* modulation of mesenchymal marker expression and stress fiber formation. It is thought that IL-24 acts on various types of tumor cells, thus our findings that suggest elucidation of IL-24 functions not mediated *via* its receptors could lead to identification of potential anticancer targets.

Authors' Contributions

Hidero Kitasato contributed to the conception and design of the study and revised the manuscript. Shotaro Maehana and Yuta Matsumoto performed the experiments. Fumiaki Kojima helped the phalloidin staining and revised the manuscript. All Authors approved the final manuscript.

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