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Short communication

Recombinant human interleukin 24 reverses Adriamycin resistance in a human breast cancer cell line

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ABSTRACT

Background: The major cause of multidrug resistance is over-expression of membrane P-glycoprotein (P-gp). We investigated the effect of recombinant human interleukin 24 (rhIL-24) on the Adriamycin (ADM)-resistant human breast cancer cell line MCF-7/ADM.
Methods: The cytotoxicity of rhIL-24 and ADM was determined by 3-[4,5-dimethylthiazol-2-yl], 5-diphenyl tetrazolium bromide (MTT) assays. The expression of P-gp was assessed by confocal microscopy and Western blot analysis.
Results: The IC_{50} values for rhIL-24 in MCF-7/wild-type and MCF-7/ADM cells were 0.17 and 14.6 μM, respectively. The IC_{50} value of Adriamycin in MCF-7/ADM cells decreased in a dose-dependent manner when rhIL-24 was used. The resistance modulating factor (RMF) was directly proportional to the dose of rhIL24. ADM accumulation increased while P-gp expression decreased at a low dose (4 μM) of rhIL24 in MCF-7/ADM cells. The expression of P-gp was decreased at 4 μM in confocal microscopy and western blot analysis.
Conclusions: rhIL-24 circumvented the drug-resistance of MCF-7/ADM cells *via* activation of the transcription factor Stat 3. rhIl24 has potential to act as a P-gp inhibitor to reverse Adriamycin resistance in breast cancer.

Introduction

Cancer multidrug resistance is the cross-resistance or insensitivity of cancer cells to the cytostatic or cytotoxic actions of various anticancer drugs [1]. Juliano and Ling [2] discovered a glycoprotein (P-gp) of 170 kDa that belongs to the superfamily of adenosine triphosphate-binding cassette transporters that are associated with the degree of drug resistance [3]. Overexpression of P-gp actively pumps drug substrates out of cancer cells thereby decreasing their intracellular accumulation [4]. This is the primary mechanism in the development of multidrug resistance (MDR) [5]. Hyaluronan (HA; a major component in the extracellular matrix of most mammalian tissues) and CD44 (a cell surface receptor that belongs to a family of multifunctional transmembrane glycoproteins expressed in mammary cells) are also involved in chemotherapeutic drug resistance. HA binding is capable of stimulating MDR1 and P-gp expression and drug resistance in breast tumor cells [6]. Therapeutics targeting HA-mediated signaling are believed to be promising anti-cancer drugs [7]. MDR is recognized as a major reason for the failure of cancer therapy, and its reversal has become one of the primary goals of modern approaches to therapy [8]. Previously, it was suggested that recombinant human interleukin 24 (rhIL-24) has clinical potential as an anti-cancer drug, and it has many advantages over existing cancer therapeutics [9]. IL-24 has two functional heterodimeric receptors, IL-20R1/IL-20R2 and IL-22R1/IL-20R2, in which R1 has a long cytoplasmic tail and R2 has a short cytoplasmic tail [10]. IL-24 preferentially signals through IL-22R1/IL-20R2 [11]. The binding of IL-24 to both receptors leads to the activation of Stat-1 and Stat-3 [10]. Stat 3 is a component of the Janus-activated kinase (JAK)/Stat signaling pathway, which plays an important role in regulating a variety of

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biological activities in normal and cancer cells [6]. The aim of the present study was to determine whether recombinant human IL-24 synergizes the anticancer effect of ADM by decreasing the Pgp expression and reverse Adriamycin resistance in tumor-resistant cells (MCF-7/ADM). The results suggest a new avenue of research for exploring the mechanisms of tumor resistance.

Materials and methods

Bacterial expression, refolding, and analysis

Escherichia coli strain BL21 (DL3) was transformed with plasmid pET21a (+), in which the IL-24 gene had been inserted between the XhoI and BamHI sites. Host cells were transformed using the $CaCl_2$ method. The rhIL-24 strain was transferred into 250 ml of modified medium and incubated with continuous shaking at 180 rpm at 37 °C for 8–10 h. The culture was transferred to 3-L fermenters containing fermentation medium. The fermentation media composition and production process described in detailed in our previous work [12]. Cells were harvested by centrifugation at 8000 rpm for 30 min. The harvested cell-paste was dissolved in 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM EDTA and 1 mM PMSF. Cells were lysed and inclusion bodies (IBs) recovered by centrifugation at 8000 rpm for 30 min at 4 °C. IBs were washed with 50 mM Tris–HCl buffer (pH 8.0) containing 5 mM EDTA and finally washed with distilled water to remove contaminating salt and detergent. IBs were dissolved in 5 ml extraction buffer I and centrifuged at 12,000 rpm for 30 min at 4 °C. The supernatant was diluted in 200 ml dilution buffer, and the precipitate was collected by centrifugation at 12,000 rpm for 30 min at 4 °C. The precipitate was further dissolved in 5 ml extraction buffer II. The solution was stirred for 20 min and centrifuged at 12,000 rpm for 30 min at 4 °C. The supernatant was collected and the total protein concentration measured by the Bradford assay. The solution was added dropwise up to 400 ml of refolding buffer and stirred slowly with a magnetic stirrer at 4 °C overnight. Refolded rhIL-24 was filtered through a 1.2 μm filter, concentrated, and buffer exchanged through a tangential flow filtration system. The refolded protein pH was adjusted to 5–6 by adding 2 M acetic acid and loaded on an SP sepharose column. SDS-PAGE (15%) was performed according to the method of Laemmli. Western blot analysis was carried out using anti-rhIL-24 antibody (Abcam, Cambridge, MA, USA). Ultra pure water was used throughout the upstream and downstream processed. Anion Exchange chromatography helps to minimize the level of endotoxin by removal of negatively charge endotoxin during purification. LAL method (Houshiji, China) was applied to detect the limit of bacterial endotoxin from rhIL24 purified product.

Cells and cell cultures

The wild-type human breast cancer cell line MCF-7/WT (sensitive cells) and the Adriamycin (ADM; doxorubicin)-resistant cell line MCF-7/ADM were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Long-term culture was performed in an incubator at 37 °C with 5% CO_2 and saturated humidity. MCF-7/ADM cells were continuously cultured in the above medium containing 1.0 μg/ml ADM to maintain the drug-resistance. Two weeks prior to experiments, MCF-7/ADM cells were placed in Adriamycin-free medium for culture.

Cytotoxicity assay

Cells were seeded onto 96-well plates at 1×10^4 MCF-7/ADM cells/well and 0.5×10^4 MCF-7/WT cells/well, and incubated in a humidified atmosphere of 5% CO_2 overnight, then rhIL24 and Adriamycin were added. For the control, medium was added instead of the drug. After 24-h incubation, 20 μL MTT (5 g/L in PBS) was added. The plates were incubated for 4 h and 200 μL DMSO/well was added. The absorbance at 570 nm was recorded using an enzyme-linked immunosorbent assay multiscan reader. The survival rate was calculated as follows:

$$\text{Survival rate } (\%) = \frac{T - B}{U - B} \times 100$$

where T represents treated (absorbance determined when tumor cells were exposed to drugs); U, untreated (absorbance of untreated cells); and B, blank (absorbance when neither the drug nor MTT was added). The IC_{50} for ADM in rhIL-24-treated and untreated tumor cells was determined and the resistance modulating fraction (RMF) was calculated as follows [13]:

$$\text{RMF} = \frac{IC_{50}\text{ADM}}{IC_{50}} \quad (\text{ADM} + \text{rhIL24})$$

In situ analysis of P-gp expression by confocal laser scanning microscopy

The MCF-7/ADM cells were cultured and prepared in confocal microscopy dishes. Growth medium was added and the cells were stored at 37 °C with 5% CO_2 for 24 h until they covered 80% of the dish. The cells were washed twice with PBS and fixed with 4% formaldehyde in 0.1 M phosphate buffer pH 7.4. After washing and blocking for 10 min, cells were permeabilized for 10 min at room temperature with 0.2% Triton-X100 in 0.1 M phosphate buffer pH 7.4. Then, the cells were washed and blocked with 1% BSA for 30 min, washed twice, incubated overnight with the P-gp antibody (1:20), and incubated with rabbit anti-mouse IgG (1:200) at room temperature for 60 min. Finally, the cells were washed and incubated with the nuclear stain DAPI (5 μg/ml) for 10 min at room temperature. The prepared cells were then observed using a laser scanning microscope (Fluoview 500; Olympus, Tokyo, Japan) with untreated cells as the control. Image processing was performed with Leica confocal microscopy software at 488 nm.

Western blot analysis of P-gp expression

P-gp expression was investigated by Western blotting. For this purpose, MCF-7/ADM cells (2×10^5/ml) were exposed to rhIL-24 (4 μM) and left at 37 °C with 5% CO_2 for 24 h. After washing, the cells were trypsinized and centrifuged at 100 rpm for 5 min, the supernatant was removed, and the cells were lysed with RIPA lysis buffer (Bayotime, China) for 20 min and centrifuged at 12,000 rpm for 5 min. Cell extracts were fractionated on an 8% sodium dodecylsulfate (SDS)-polyacrylamide gel and then electro-transferred to nitrocellulose membrane. The membrane was blocked with 5% fat-free milk-powder in 50 mmol/L Tris-buffered saline (TBS), pH 7.6, with 0.1% Tween-20 (TBS-Tw20) at room temperature for 1 h. The membrane was washed three times and incubated overnight at 4 °C with the mouse monoclonal antibody to P-gp (C219; AbCam, Inc., Cambridge, MA) and the lower part of the membrane with the rabbit monoclonal anti-β-actin antibody (Cell Signaling Technologies, Danvers, MA) at 1:1000 dilution in TBS-Tw20 containing 5% fat-free milk powder. After this step, the membrane was washed three times with TBS-Tw20 and reacted for 1 h with the secondary antibody, anti-mouse or anti-rabbit IgG (Cell Signaling Technologies) conjugated with HRP. The bound antibodies were detected with an ECL Western blotting system (GE Healthcare), and the chemiluminescent signals were detected