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Bacterial redox protein azurin induce apoptosis in human osteosarcoma U2OS cells

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Abstract

As a low molecular weight redox protein elaborated from the pathogenic bacteria *Pseudomonas aeruginosa*, azurin is one of representative bacterial products applied in the treatment of tumour. We found that the growth of U2OS cells was significantly inhibited by azurin in a dose-dependent manner with the IC_{50} value of $114.54 \pm 7.65\ mg\ l^{-1}$. But the growth of MG63 cells or L02 cells was almost not inhibited by azurin ($P < 0.05$). Moreover, when treated with azurin, U2OS cells showed typical apoptotic morphological features observed by fluorescent microscopy (AO and Hoechst 33258) and transmission electron microscopy. Typical DNA "ladder" bands were also observed. The apoptosis rate was 35.8% tested by fluorescence-activated cell sorter (Annexin-V-$FITC^+/PI^-$) and the cell-cycle arrested in G_1 phase. But no apoptotic features were observed in control cells. The down-regulation of Bcl-2 (an inhibitor of apoptosis) were detected in U2OS cells when azurin was added for 24 h. In contrast, the level of Bax and caspase-3 were significantly up-regulated. So we concluded that azurin could selectively induce apoptosis of human osteosarcoma U2OS cells and the induction of apoptosis by azurin was closely associated with down-regulation of Bcl-2, up-regulation of Bax and activation of caspase-3.
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Keywords: Azurin; Osteosarcoma; Apoptosis; Bax; Bcl-2; Caspase-3

1. Introduction

Osteosarcoma is a malignant tumour of bone that is most prevalent in adolescents and young adults. Osteosarcoma accounts for approximately 5% of the tumours in childhood and 80% of this tumour originates around the knee [1]. Although adjuvant chemotherapy is effective in improvement of patient survival and treatment of the primary tumour [2], some groups of patients who present with metastatic disease and patients with tumours that recur after treatment continue to have a poor prognosis. In addition, the frequent acquisition of drug-resistant phenotypes and the occurrence of "second malignancy" are often associated with chemotherapy, which remains as serious problems. Therefore, there is a clear need for newer effective agents for patients with osteosarcoma, especially for patients who present with metastatic disease or develop disease recurrence.

Recently, live or attenuated pathogenic bacteria or their products were used in the treatment of cancer [3–5]. A significant regression of subcutaneous tumours in mice was observed by combining anaerobic bacteria with various chemotherapeutic agents [6].

Azurin, a cupredoxin type of electron transfer and purified low molecular weight redox protein elaborated from the pathogenic bacteria *Pseudomonas aeruginosa*, has been reported to induce and trigger apoptosis in several human cancer cells selectively. These findings in vitro have been confirmed in nude mice bearing tumour xenograft in vivo. Furthermore, it is completely lack of toxicity. At necropsy, none of the treated mice showed any histological evidence of toxicity and all of the viscera were within normal limits

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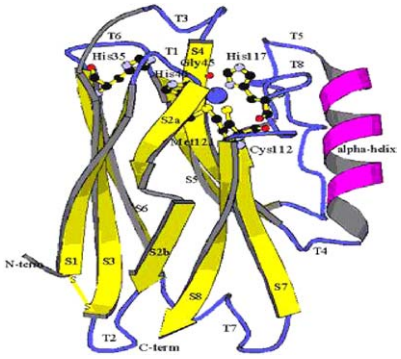
[7–9]. However, as a potent inducer of apoptosis, the precise mechanism of azurin-induced apoptosis is still unclear.



The protein p53 has an important role in pathogenesis of neoplasia [10]. The mechanism involved entails a rapid increase in p53 protein levels and the mediation of several cellular responses including G_1 arrest, DNA damage repair and induction of apoptosis [11]. One of the major signaling pathways involved in apoptotic cell death includes the intracellular caspases, a family of structurally related cysteine proteases [12]. Caspase activity is responsible, either directly or indirectly, for the cleavage of cellular proteins, which are characteristically proteolyzed during apoptosis. For example, caspases-2, -3, -6, -7 and -9 can cleave poly(ADP ribose) polymerase (PARP) [13]. Bcl-2 family proteins are one of the already identified regulators of apoptosis. Bcl-2 family of homologous proteins represents a critical checkpoint within most apoptotic pathways, acting upstream of such irreversible damage to cellular constituents [14]. At least 15 Bcl-2 family members have been identified so far in mammalian cells. They function either as pro-apoptotic (Bax, Bak and Bad) or anti-apoptotic (Bcl-2 and Bcl-XL) regulators. These proteins form heterodimers of anti- and pro-apoptotic members, thereby titrating one another's function [14]. The ratio of anti- and pro-apoptotic proteins determines in part how cells respond to apoptotic or survival signals [15]. Despite extensive analysis of anti-tumour activities of azurin, its ability to modulate osteosarcoma growth has not yet been well characterized. We used osteosarcoma cell lines to study the effect of different concentrations of azurin on cell viability and genes related to apoptosis. Our results demonstrated that azurin selectively causes growth arrest and apoptosis in U2OS cells and the growth inhibitory effects of azurin appeared to be mediated by the regulation of Bcl-2, Bax and caspase-3. Azurin-induced apoptosis and the induction may be related to p53 status of the cell lines.

2. Materials and methods

2.1. Chemical reagents

Azurin, purchased from Sigma (MO, USA), was dissolved in RPMI-1640 or MEM culture medium with the final concentration of $1\,mg\,l^{-1}$ and was stored in a dark-coloured bottle at 4 °C as a stock solution, The stock was diluted to the required concentration immediately before use with growth media. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), AO, Hochest33258 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Primary antibodies to caspase-3, Bcl-2, Bax and β-actin were from Neomarkers (Fremont, CA, USA). Western blot chemiluminescent detection system (LumiGLO System) was purchased from KPL (Gaithersburg, MD, USA). All other regents are analytical or cultured grade purity.

2.2. Cell culture

The human osteosarcoma cell line U2OS and MG63 (ATCC, CRL-1427, HTB 96, Manassas, VA, USA) and the human hepatocyte L02 (a normal liver cell line, was provided by Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science, China) were used for this study. Cell lines U2OS and L02 are p53 wild type and MG63 is devoid of endogenous p53. The cells were cultured in RPMI-1640 (U2OS and LO2 cells) and MEM (MG63 cells) culture medium (Gibco, NY) at 37 °C in a humidified incubator with a 5% CO_2 atmosphere. The culture medium was supplemented with 0.03% L-glutamine (Gibco) and 10% fetal bovine serum (FBS), penicillin ($50\,U\,ml^{-1}$) and streptomycin ($50\,g\,ml^{-1}$).

2.3. Azurin treatment of cells

The cells were exposed to azurin at different concentrations ($0–500\,mg\,l^{-1}$) and for different time (0–72 h). Cells grown in media containing equivalent amount of RPMI-1640 or MEM without azurin served as control.

2.4. Cell growth assay and IC_{50} value by MTT method

The cytotoxic effect of azurin on U2OS, L02 and MG63 cells were assessed by MTT assay, which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. The cells were suspended in 96-well plate (Coastar from Corning, NY) at a density of 2×10^4 cells per well. After 24 h, they were treated with various concentrations ($0–500\,mg\,l^{-1}$) of azurin for different time intervals (0–72 h). Four hours before the end of incubation, 20 μl of MTT solution ($5 \times 10^3\,mg\,l^{-1}$) was added to each well. The supernatant was removed and 150 μl DMSO was added to each well. An ELISA reader was used to measure the absorbance at 525 nm and IC_{50} value was assessed by Bliss method.

2.5. Morphological studies of apoptotic cell

2.5.1. Fluorescence microscopy observation

U2OS cells exposed to different concentrations of azurin for overnight were harvested by centrifugation, washed with

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