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Expression of 14-3-3δ, cdc2 and cyclin B proteins related to exotoxin A-induced apoptosis in HeLa S_3 cells

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Abstract

After reports on regression of cancer in humans and animals infected with microbial pathogens date back more than 100 years, much effort has been spent over the years in developing wild type or attenuated bacterial and purified bacterial proteins for the treatment of cancer. *Pseudomonas aeruginosa* exotoxin A (ETA) is known to inhibit cell growth and trigger significant cell death in various cancer cells. Although ETA induces apoptosis of cancer cells, its exact mechanism of action is not yet known. Four different assays were performed in this study: morphological assessment of apoptotic cells, cell cytotoxicity, cell cycle analysis and Western blot analysis. The proliferation and survival in the cells treated with ETA was decreased. In addition, percentages of apoptotic HeLa S_3 cells treated with ETA were increased. ETA-induced apoptosis rates were confirmed to have increased in a dose-dependent manner through annexin V binding assay. Flow cytometric analysis was examined to ascertain whether ETA could regulate cell cycle in HeLa S_3 cells. ETA treatment demonstrated that the expression of 14-3-3δ proteins was increased, while expression of cdc and cyclin B proteins was decreased, suggesting that ETA induces cell cycle arrest and then progresses to apoptosis. Therefore, these results suggest that *P. aeruginosa* ETA induced apoptosis in HeLa S_3 cells.

Keywords: *Pseudomonas aeruginosa* exotoxin A; 14-3-3δ; cdc; Cyclin B

1. Introduction

Many bacteria have been used in an effort to reduce the growth rate or size of tumors. Bacteria are able to trigger apoptosis by a whole variety of mechanisms including the secretion of protein synthesis inhibitors, pore-forming proteins, and molecules activating the endogenous death machinery in the infected cell or lipopolysaccharides and other superantigens [18]. Current chemotherapeutic approaches for cancer are in part limited by the inability of drugs to destroy neoplastic cells within poorly vascularized compartments of tumors. Vogelstein and coworkers have created a new strain of anaerobic bacteria, devoid of its toxic genes, that lead to dramatic and prolonged regression of subcutaneous tumors when systematically administered with conventional drugs [6].

Pseudomonas aeruginosa produces several extracellular products that after colonization can cause extensive tissue damage, bloodstream invasion, and dissemination. Exotoxin A (ETA), a 66-kDa protein of 613 amino acids, is considered to be the most toxic factor secreted by *P. aeruginosa*. Because of its potent cytotoxicity, ETA has been widely used to generate fusion proteins to

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kill target cells [4]. The toxin enters into eukaryotic cells via receptor-mediated endocytosis, and then it is cleaved in the endocytic vesicles and translocated into the cytoplasm, where it catalyzes the transfer of the ADP-ribosyl moiety of NAD^+ to elongation factor 2 (EF-2). Inactivation of EF-2 leads to the inhibition of protein synthesis and to host cell death. Studies performed in the early 1990s reported that ETA, as well as other inhibitors of protein synthesis, induced apoptosis of mammalian cells characterized by chromosomal DNA degradation into oligonucleosome-sized fragments, chromatin condensation and cell nuclei fragmentation [12].

Apoptosis or programmed cell death is known as an important biological mechanism that contributes to the maintenance of the integrity of multicellular organism [10]. It is induced by a wide variety of cellular stresses such as DNA damage, UV radiation, ionizing radiation and oxidative stress [13], and is morphologically distinct from necrosis in many of its characteristic changes as follows: DNA fragmentation, chromatin condensation [19], cytoplasmic membrane blebbing, and cell shrinkage. Antitumor agents also induce apoptosis in some cancer cells both in vitro and in vivo, indicating that apoptosis plays a very important role in cancer chemotherapy.

Annexin V, belonging to a recently discovered family of proteins, the annexins, with anticoagulant properties has proven to be a useful tool in detecting apoptotic cells since it preferentially binds to negatively charged phospholipids such as phospholipid-like phosphatidylserine (PS) in the presence of Ca^{2+} and shows minimal binding to phosphatidylcholine and sphingomyeline. Changes in PS asymmetry, which is analyzed by measuring annexin V binding to the cell membrane, were detected before morphological changes associated with apoptosis have occurred and before membrane integrity has been lost. By conjugating FITC to annexin V, it is possible to identify and quantitate apoptotic cells on a single-cell basis by flow cytometry. Staining cells simultaneously with FITC–annexin V (green fluorescence) and the non-vital dye propidium iodide (red fluorescence) allows (bivariate analysis) the discrimination of intact cells ($FITC^-PI^-$), early apoptotic ($FITC^+PI^-$) and late apoptotic or necrotic cells ($FITC^+PI^+$).

Diverse death signals activate different pathways that converge toward a conserved execution machinery composed of specific apoptotic proteases [7]. The hierarchical activation of these proteases provokes the cleavage of key nuclear and cytoplasmic proteins as well as the activation of DNases, leading to the nuclear and cytoplasmic lesions characteristic of apoptotic cell death [15]. Although the pathways leading to apoptosis are not fully elucidated, several genes that play a role in the process have been identified, and some, such as *p53*, *14-3-3δ*, *cdc2*, and *cyclin B* have an important role in cell cycle. *14-3-3σ* is involved in cell-cycle control and prevents the accumulation of chromosomal damage. Elevated levels of several 14-3-3 isoforms have been found in cancers and cell lines derived from tumors [16]. *p53* either induces cell-cycle arrest at G_1 or G_2 by increasing the transcription of *p21* gene or initiate apoptosis, which protects the organism by eliminating the defective cell [11]. On the other hand, *p53* activates the transcription of *14-3-3σ* gene and decrease *cdc2* expression.

Because ETA was known to be the most toxic exoproduct of *P. aeruginosa*, this work was performed to investigate its apoptotic effect on HeLa S_3 cells. Although much has been learned concerning the metabolism of ETA, a clear understanding of the biochemical bases for its apoptosis-inducing activity has not yet emerged. Thus, this study was performed about the expression of 14-3-3δ, cdc2 and cyclin B proteins related to ETA-induced apoptosis in HeLa S_3 cells.

2. Materials and methods

2.1. Cell culture

HeLa S_3 cells were used throughout this investigation. Monolayer cultures of these cell lines were grown at 37 °C in humidified 5% CO_2 incubator using Eagle's minimum essential medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% newborn calf serum and gentamycin (50 μg/M∕).

2.2. MTT assay

The cytotoxic effect of ETA (sigma) in cells was estimated by MTT assay. In the MTT assay, cells were placed in a 96-well plate and incubated for 24 h. Then cells were treated with various concentrations of ETA for 24 h. And then, the cells were treated with 1 mg/M∕ of MTT in the growth medium. Cells were incubated at 37 °C, 5% CO_2 for 4 h. The medium was aspirated and the formazan crystals, which are formed from MTT by NADH-generating dehydrogenases in metabolically active cell, were dissolved in 200 μl DMSO. Cell viability was evaluated in comparison to the control culture (taken as 100%) by measuring the intensity of the blue color (OD at 570 nm) by a multi-well reader (Quant, Bio-Tek, Highland Park, USA). The assay was performed in triplicate.

2.3. Cell viability assay

Cell viability was measured by hemocytometer using the trypan blue dye exclusion. Trypsinized cells were incubated with 0.4% trypan blue solution (Sigma) for 10 min, and more