RESEARCH ARTICLE

Correlation between E-Cadherin-Regulated Cell Adhesion and Human Osteosarcoma MG-63 Cell Anoikis

Ding-Sheng Lin*, Le-Yi Cai, Jian Ding, Wei- Yang Gao

Abstract

Purpose: The aim of this study was to investigate the relationship between cell adhesion and anoikis evasion among human osteosarcoma cells (MG-63), and to further study the molecular mechanisms. Materials and Methods: Human osteosarcoma cells (MG-63) were assessed for apoptosis, and caspase-3, E-cadherin and β-catenin expression in EDTA and control non-EDTA groups. Results: MG-63 cells were predominantly aggregated when in suspension, and the suspended cells were more dispersed in the EDTA group. Following culture in suspension for 24 h, 48 h, or 72 h, the rates of apoptosis were 34.88%±3.64%, 59.3%±7.22% and 78.5%±5.21% in the experimental group and 7.34%±2.13%, 14.7%±3.69%, and 21.4%±3.60% in the control group, respectively. Caspase-3 expression progressively increased and E-cadherin and β-catenin were decreased in the experimental group, whereas there was no change in the control group. Conclusions: MG-63 cells could avoid anoikis through cell adhesion, and E-cadherin might play a role in this process.

Keywords: Osteosarcoma - anoikis - cell adhesion - E-cadherin

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Introduction

Anoikis is a specific form of programmed cell death, initially described as a form of apoptosis in 1994 by Frisch, who suggested that the survival of normal epithelial or endothelial cells were adhesion dependent; their survival was thought to depend upon intercellular and cell-matrix signals and they were therefore termed "anchorage dependent." If they lost intercellular contact and matrix support, for example, if the normal epidermal cells or non-metastatic cancer cells shed from their original location and entered the bloodstream, apoptosis was thought to occur. "Anoikis" was used to describe this specific instance of apoptosis, where cells detached from their original environment (Lee and Kim, 2013; Chang et al., 2014; Chaotham et al., 2014).

Anoikis is important as it can prevent detached cells from implanting and growing in inadequate environments (Kanda et al., 2014). However, many malignant cells, which are capable of metastasis, can avoid anoikis. They do not undergo apoptosis after shedding from the tumor body and entering the circulatory system, where they migrate to the other locations and divide. This avoidance of anoikis could be fundamental to tumor metastasis. Anoikis avoidance of malignant tumor cells has been proven in vitro using many different cancer cells, such as liver cancer, colorectal cancer, glioma, metastatic bone cancer, and oral squamous cell carcinoma cells (Cao et al., 2014; Fan et al., 2014; Maroni et al., 2014; Sliginer et al., 2014).

Cell adherence and intercellular signal transduction help define the biological characteristics, growth, apoptosis, and division of cells, but they also have an important effect on the evasion of anoikis, invasiveness, and metastasis of tumor cells (Burnette et al., 2014). Studies have shown that when cells detach from the matrix and become aggregated, the cells can escape anoikis by intercellular signaling, which mimics cell-matrix signal transduction (Ivanova et al., 2013; Paoli et al., 2013). Aggregation is also believed to enhance their resistance to chemotherapeutic drugs.

Osteosarcoma is a clinically common disease, but the mechanisms of its occurrence and metastasis are not well understood (He et al., 2014). Our previous work has shown that human MG-63 osteosarcoma cells form large and dense aggregates in suspension, without significant apoptosis-a feature of anoikis evasion (Lin et al., 2008; Lin, 2012). Following suspension, the osteosarcoma cells adhere to each other and form clumps, which simulates the environment of cell-matrix adhesion, and allows the osteosarcoma cells to avoid anoikis. However, the features of this relationship between anoikis avoidance and cell adhesion are not clear. In this study, we used the EDTA to block the adhersion of the osteosarcome cell to explore the correlation between cell adhesion and anoikis avoidance in osteosarcoma cells.

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Materials and Methods

Preparation of cell suspension culture plates

Poly (2-hydroxyethyl methacrylate) (poly-HEMA; Sigma, St. Louis, MO, USA) (2 ml of a 50 mg/ml solution) was added to the wells of a six-well plate (Hangzhou Evergreen Biological Engineering Company, China), and the plate was left at room temperature to completely dry. The above process was repeated once, and 1× phosphate-buffered saline (PBS) was used to wash the six-well tissue culture plate four times. The plate was then irradiated with ultraviolet light on a clean bench and stored for later use.

Cell treatment

Cell digestion was accomplished with 0.25% trypsin containing 0.01% ethylenediaminetetraacetic acid (EDTA; Sigma, St. Louis, MO, USA), and a 5×10^5 cells/ml single-cell suspension was prepared. The cell suspension (2 ml) was added to the poly-HEMA-treated six-well plate, and these were set as the experimental group. Simultaneously, 2 ml of non-EDTA-treated cells were inoculated into an identical six-well plate and set as the negative control. The cells were incubated at 37°C with 5% CO_2 for 24 h, 48 h, 72 h, or 7 days.

Observation of cellular morphology

Cells (5×10^5) obtained from the experimental and control groups were resuspended in 2 ml of culture solution. A light microscope (Philips Tecnai 10, Philips Co., Amsterdam, the Netherlands) was used to observe cellular morphology and cell colony formation.

Flow cytometry

Cellular suspensions (5×10^5 cells/ml) were obtained and fixed with 70% ice cold ethanol, and maintained at 4°C for 24 h. Cells were subsequently washed twice with PBS. RNase A (200 μl of a 1 g/L solution) was added, and the cells were incubated at 37°C in a water bath for 30 min, followed by incubation with prodidium iodide staining solution in the dark for 30 min. A FACScan flow cytometer (Becton Dickinson, USA) was used for data collection. The rate of apoptosis was calculated using CellQuest software.

Western blot

Cells were collected and dissolved in 1 ml RIPA lysis buffer (50×10^{-3} mol/L Tris-HCl [pH 7.4], 150×10^{-3} mol/L NaCl, 1% NP40, 5×10^{-3} mol/L EDTA, 5×10^{-3} mol/L NaF, 2×10^{-3} mol/L Na_3VO_4, 1×10^{-3} mol/L PMSF, 5 μg/ml leupeptine, and 5 μg/ml aprotinin). The Bradford method was used to quantify protein concentration, and equal quantities of protein from each treatment group were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Bio-Rad California, USA). Separated proteins were transferred onto polyvinylidene difluoride membranes and blocked overnight with 5% skimmed milk powder. Caspase-3, β-catenin, and E-cadherin monoclonal antibodies (CST, USA) and β-actin (1:1000; Sigma, USA) were added for 12 h with shaking, after which, membranes were washed three times, for 10 min each, with PBS with Tween 20. Then, anti-murine IgG horseradish peroxidase-conjugated antibody was added and incubated for 2 h on a shaker. Membranes were then washed with PBS with Tween 20 three times, incubated with ECL reagent, and dried. Proteins were detected on Kodak X-ray films. Semi-quantitative analysis of band grey values was conducted using Leica Q500iw image analysis software.

Fluorescent quantitative reverse transcription (RT)-polymerase chain reaction (PCR)

Trizol reagent (1 ml) (Shanghai Biological Engineering Co., China) was added to the cells (5×10^8 cells/L), and total RNA was extracted according to the manufacturer's protocol. Absorbance at 260 nm and 280 nm was used to estimate the purity of extracted RNA. The RT-PCR reaction (50 μL) mix contained 5 μL 10× PCR buffer (containing 20 mmol/L Mg_{2+}), 4 μL dNTPs (containing 2.5 mmol/L dATP, dGTP, dCTP and dTTP), 1 μL of forward and reverse primers (10 μmol/L), 0.5 μL 5 IU Taq polymerase, 4 μL serum, and 34.5 μL of ultrapure water. The cycling parameters of the RT-PCR reaction were as follows: denaturation at 95°C for 5 min; 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. The results were analyzed using SDS7000. The threshold value was set within the range of product index growth, the CT value was determined, and the results were obtained by comparing the CT value with the internal reference, GAPDH. The primers used were as follows: human β-catenin - sense primer 5'-GATTTGATGGAGTTGGACATGG-3', antisense primer 5'-TGTTCTTGAGTGAAGGACTGAG-3'; human caspase-3-sense primer 5'-GAAGCGAATCAATG GACTCTG-3', antisense primer 5' GCACAAAGCGACT GGATGAA-3'; human E-cadherin - sense primer 5'-ATCAAAGGTATCACGGCAAACG-3', antisense primer 5'-CGGAGAGCTCGTCCACGTAT-3'; and human GAPDH-sense primer 5'-ACCTGACCTG CCGTCTAGAA-3', antisense primer 5'-TCCACCAC CCTGTTGCTGTA-3'.

Statistical analysis

All data are expressed as mean±standard deviation. Single factor analysis of variance was used to determine the difference between the control and experimental groups, with $p<0.05$ considered as statistically significant.

Results

Morphology of suspended osteosarcoma cells

In suspension, the MG-63 osteosarcoma cells formed large, dense aggregates with a few scattered cells. In the EDTA group, the osteosarcoma cells were much more dispersed, with fewer dense aggregates (Figure 1).

Apoptosis rates

In the suspension culture, the rate of MG-63 apoptosis was significantly higher in the EDTA experimental group than in the control group. After culturing in suspension for 24 h, 48 h, and 72 h, the rates of apoptosis were 34.88%±3.64%, 59.30%±7.22%, and 78.50%±5.21% in the EDTA experimental group and 7.34%±2.13%, 14.65%±3.69%, and 21.41%±3.60% in the control group,

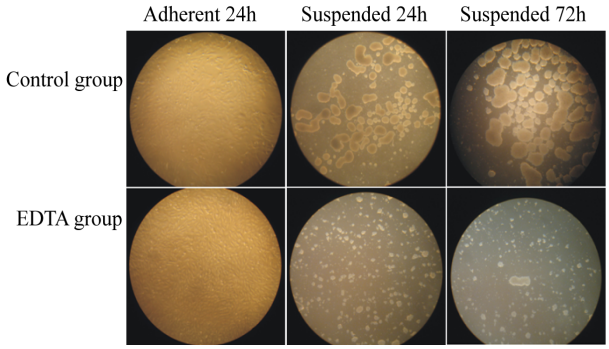

Figure 1. Morphological Features of MG63 Osteosarcoma Cell Suspension Cultures Under Light Microscopy (×40)

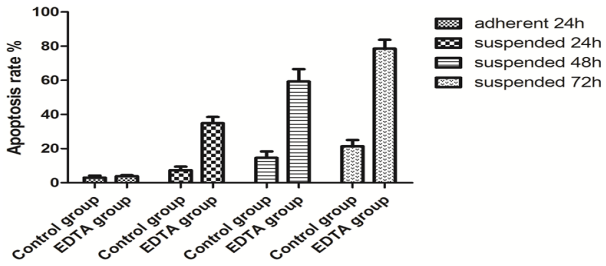

Figure 2. Apoptosis Rates of Adherent and Suspended Cell Growth at Different Times

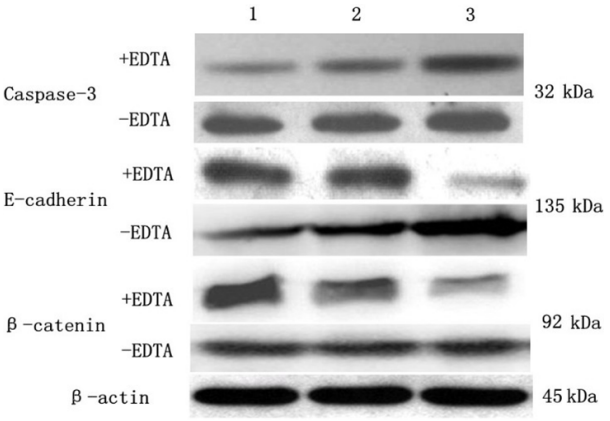

Figure 3. Caspase-3, β-Catenin and E-Cadherin Protein Expressions in EDTA and non-EDTA Suspension-Cultured MG63 Osteosarcoma cells - (1: Suspended for 24 hr, 2: Suspended for 48 hr, 3: Suspended for 72 hr)

respectively (Figure 2).

Protein expression and gene transcription levels

The protein expression and gene transcription levels of caspase-3 showed a time-dependent growth in the EDTA group, while those in the non-EDTA group showed no significant time dependence. The protein expression and gene transcription levels of E-cadherin gradually decreased with time in the EDTA group, while these levels increased with time in the non-EDTA group. The protein expression and gene transcription levels of β-catenin showed a time-dependent decrease in the EDTA group, whereas no significant changes were observed in the non-EDTA group (Figures 3 and 4).

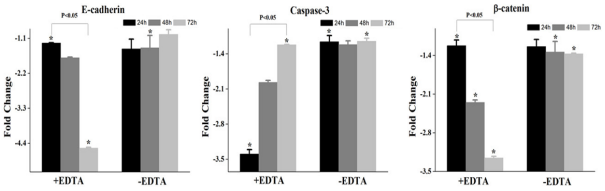

Figure 4. Gene Expression Levels of Caspase-3, β-Catenin and E-Cadherin in the EDTA-Added and Non-EDTA-Added Suspension-Cultured MG63 Osteosarcoma Cells

Discussion

Anoikis plays a role in the occurrence, development, regeneration, and degradation of an organism. It is important for the maintenance of structural integrity and retaining the balance of the internal tissue environment. Furthermore, anoikis has been shown to occur in multiple tumor types. Cadherin is a type of cell surface protein, which regulates isoantigens and calcium-dependent cell-cell adhesion (Asiaf et al., 2014); it has been shown to be very important for cellular structure and differentiation (Frisch and Screaton, 2001; Marco et al., 2003). Studies have shown that the E-cadherin signaling pathway can inhibit anoikis avoidance in Ewing's sarcoma (Kang et al., 2007; Strauss et al., 2010).

In the experimental group of our study, the calcium chelator, EDTA, was added to inhibit the effect of cadherin (Ma et al., 2010). The cells in this treatment group were more loosely connected than in the control group, forming fewer cell aggregations, and the rate of apoptosis was higher. This suggests that osteosarcoma cells can simulate the cell-matrix adhesion environment through intercellular adhesion, thus generating anti-anoikis signaling. Similar adhesion in tumors could enable osteosarcoma cells to avoid anoikis. While another study showed that, MicroRNA-218 could inhibit the growth of osteosarcoma, and the mechanism might be through reducing the tumor growth-related genes TIAM1, MMP2 and MMP9 (Jin Jet al., 2013), thus carried out the study from the views of cytoadherence and tumor metastasis. But the impacts of MicroRNA towards the anoikis of osteosarcoma still needed the further studies from the genetic aspects.

E-cadherin (Tu and You, 2014) and β-catenin (Perry et al., 2011) are important intracellular signaling molecules associated with cell anchorage. They transduce cell proliferation and transformation signals through the phosphorylation and activation of PI3K/AKT, thereby inhibiting apoptosis. Zhang et al. (2014) found when studying the non-hodgkin lymphoma that the activation of Wnt/β-catenin signal pathway could induce the downregulation of β-catenin and high expression of E-cadherin thus reducing the expressions of cytoadherence and tumor invasion-related proteins, which finally could inhibit the growth of tumors.We found that E-cadherin and β-catenin gene expression and protein translation were at lower levels in the experimental group than in the control group. However, the expression of caspase-3 was increased in the experimental group compared to the control group. Caspases constitute the dominant pathway through which apoptosis occurs. Activated

caspases can induce apoptosis through specific cleavage of their substrates; caspase-3 acts as an important "executioner" factor for the classical caspase-dependent apoptotic pathway (Wang et al., 2014). Our experiments show that the calcium chelator EDTA can reduce the expression of E-cadherin and β-catenin, responsible for cell-cell anchorage, leading to a reduction in intercellular adhesion and an increased rate of apoptosis. This increased apoptosis could occur through stimulation of caspase-3, which could occur through a number of mechanisms. Further studies would be required to determine the specific mechanism through which this occurs.

Studies have shown that alteration of tumor adhesion molecule expression is an effective means of avoiding anoikis (Hynes, 1992; Regezi et al., 2002). Changes in the expression of adhesion proteins can affect downstream signaling cascades and enhance cellular survival. Many tumor cells can switch on/off the expression of adhesion molecules so that they express the correct adhesion molecules in different microenvironments, thus preventing the induction of cellular death (Su et al., 2014). In this study, EDTA was used to block intercellular adhesion and osteosarcoma anoikis was found to increase.

Increasing evidence suggests that an absence of E-cadherin is important in anoikis evasion. E-cadherin is a type I cadherin, which forms connections between adjacent cells and regulates intercellular contact. In breast cancer (Onder et al., 2008), gastric cancer (Tamura et al., 2000), and other tumors, an absence of E-cadherin has been shown to lead to anoikis evasion, which can facilitate tumor spread. Although the results of our study confirm this, further studies are required to determine the specific mechanism and whether this is dependent on PI3K/AKT. In light of our, and others, findings, it is anticipated that the E-cadherin signaling pathway could become a novel therapeutic target in cancer therapy. Anoikis-sensitizing drugs could provide an effective means of treating aggressive tumors.

He et al. (2013) performed the osteosarcoma genomic study, and found that among the Chinese population, the rs7646409 CC genotype of PIK3CA gene might increase the risk of osteosarcoma. So the investigation towards the specific mechanisms between anoikis and osteosarcoma would be an important step for the future effective treatment and prevention of osteosarcoma. Meanwhile, how to converse the laboratory research into the clinical use would also be the direction of our continuing researches.

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