Chick Embryo Extract Demethylates Tumor Suppressor Genes in Osteosarcoma Cells

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Abstract

Background Epigenetics is the study of changes in gene expression or cellular phenotype caused by mechanisms other than changes in the underlying DNA sequence. It is widely accepted that cancer has genetic and epigenetic origins. The idea of epigenetic reprogramming of cancer cells by an embryonic microenvironment possesses potential interest from the prospect of both basic science and potential therapeutic strategies. Chick embryo extract (CEE) has been used for the successful expansion of many specific stem cells and has demonstrated the ability to facilitate DNA demethylation.

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Questions/purposes The current study was conducted to compare the status of DNA methylation in highly metastatic and less metastatic osteosarcoma cells and to investigate whether CEE may affect the epigenetic regulation of tumor suppressor genes and thus change the metastatic phenotypes of highly metastatic osteosarcoma cells.

Methods K7M2 murine OS cells were treated with CEE to determine its potential effect on DNA methylation, cell apoptosis, and invasion capacity.

Results Our current results suggest that the methylation status of tumor suppressor genes (p16, p53, and E-cadherin) is significantly greater in highly metastatic mouse osteosarcoma K7M2 cells in comparison with less metastatic mouse osteosarcoma K12 cells. CEE treatment of K7M2 cells caused demethylation of p16, p53, and E-cadherin genes, upregulated their expression, and resulted in the reversion of metastatic phenotypes in highly metastatic osteosarcoma cells.

Conclusions CEE may promote the reversion of metastatic phenotypes of osteosarcoma cells and can be a helpful tool to study osteosarcoma tumor reversion by epigenetic reprogramming.

Clinical Relevance Demethylation of tumor suppressor genes in osteosarcoma may represent a novel strategy to diminish the metastatic potential of this neoplasm. Further studies, both in vitro and in vivo, are warranted to evaluate the clinical feasibility of this approach as an adjuvant to current therapy.

Introduction

Epigenetics (Greek: *επι*- over, above, outer) is the study of changes in gene expression or cellular phenotype caused by mechanisms other than changes in the underlying DNA

sequence. Epigenetics has thus been called “the code outside the code.” Examples of epigenetic modification include DNA methylation and histone modification, both of which regulate gene expression but do not alter the genetic code. For somatic stem cells, epigenetic changes in response to environmental stimuli are important to regulate stem cell function and differentiation [28, 34]. For tumor cells, the epigenetic silencing of tumor suppressor genes is associated with tumor formation and progression [2, 9, 14]. Epigenetic reprogramming of somatic cells to attain stem-like properties has been experimentally achieved by exposure of cells to an embryonic microenvironment. This may be achieved with exogenous embryonic factors such as the extract from embryonic stem cells or germinal cells [6, 35]. Similarly, exposure to an embryonic microenvironment can also exert a profound effect by epigenetically reprogramming tumor cells [20]. For example, when metastatic melanoma cells were injected into chicken or mouse embryos, the tumorigenicity and metastatic phenotypes of tumor cells were found to be suppressed [11, 27]. Amphibian oocyte extracts [1] and zebrafish embryo extracts [8] were found to repress growth and induce apoptosis of breast cancer cells and colon cancer cells, respectively.

DNA methylation occurs when a methyl group becomes fixed to a particular segment of DNA, which alters translation of that sequence. Methylation effectively turns off the translation of a particular sequence, leading to lower gene expression. DNA methylation is important in cancer. Healthy cells demonstrate methylation of repetitive sequences, whereas housekeeping/tumor suppressor genes remain unmethylated. Conversely, cancer cells undergo DNA hypomethylation of repetitive DNA sequences and hypermethylation of tumor suppressor genes associated with transcriptional silencing of these loci. Thus, even if the tumor suppressor gene is functional, it is still under-expressed because the transcription machinery does not “see” the methylated sequence. Indeed, DNA demethylation of hypermethylated tumor suppressor genes has been implicated as a key mechanism to reverse tumorigenicity of cancer stem cells [1, 20].

Chick embryo extract (CEE) is a medium component prepared from whole chicken embryos that has been specifically used for the cultivation of some stem cells such as neural crest stem cells [33] and neuroepithelial stem cells [23]. CEE provides an essential source of growth factors for stem cells [16, 33]. Previous data from our group demonstrated that CEE is necessary for the successful expansion of highly regenerative muscle-derived stem cells [16]. CEE promoted DNA demethylation, specifically on CpG islands [22]. CpG island hypermethylation of tumor suppressor genes is known to be a feature of many tumor cells [13, 17]. We suggest that CEE may generate an embryonic microenvironment for cancer stem cells [8]. It is therefore logical

to expect that CEE treatment of cancer stem cells may generate epigenetic changes, including DNA demethylation of tumor suppressor genes in tumor cells.

Osteosarcoma is the most common primary malignancy of bone [5, 7, 19], and the overall survival for patients without radiographically detectable metastases is only 65% to 70% [5, 7, 15, 29, 30]. However, the prognosis of patients with detectable metastatic disease at the time of diagnosis is only 15% to 30% survival. Because nearly all metastatic disease is to the lungs, the presence of pulmonary metastases ultimately determines patients’ outcomes.

K7M2 and K12 are related murine osteosarcoma cell populations derived from the same spontaneously occurring osteosarcoma in a Balb-C mouse. K7M2 cells are violently metastatic to the lungs and were clonally derived from the much less metastatic K12 cells [25]. K7M2 and K12 cells are thus very similar genetically and represent excellent tools to study the molecular mechanisms that regulate metastatic potential.

In the current study, we compared the status of DNA methylation of important tumor suppressor genes between K7M2 and K12 cell and investigated whether CEE treatment of K7M2 cells could cause demethylation of these tumor suppressor genes. We also investigated if the cells’ metastatic potential could be consequently reversed through CEE treatment.

Methods and Materials

Osteosarcoma Cells and Chick Embryo Extract Treatment

K7M2 and K12 are related murine osteosarcoma cell populations with differing metastatic potentials: K7M2 is highly metastatic to the lung but K12 is much less metastatic [25]. K7M2 cells and K12 cells were cultured with proliferation medium (PM; DMEM with 10% fetal bovine serum [FBS] and 5% penicillin and streptomycin). Zero percent, 2%, and 4% of CEE (Gemini Bio Products, West Sacramento, CA, USA) in proliferation medium (10% FBS in DMEM) was used for treatment of osteosarcoma cells. Cells were incubated with CEE for 2 or 4 days before the cells being fixed for observation or being harvested for mRNA isolation.

Extraction and Sodium Bisulfite Modification of Genomic DNA

Genomic DNA was isolated from osteosarcoma cells with a Qiagen Blood and Cell Culture DNA kit (Qiagen, Valencia, VA, USA) and stored at -20°C before use [10]. DNA was

Table 1. Primer sequences

Genes	Sequences
p16 methylated DNA	Forward: CGATTGGGCGGGTATTGAATTTTCGC Reverse: CACGTCATACACACGACCCTAAACCG
p16 unmethylated DNA	Forward: GTGATTGGGTGGGTATTGAATTTTGTG Reverse: CACACATCATACACACAACCCTAAACCA
p16 mRNA	Forward: CAACGCCCCGAACTCTTTC Reverse: GCAGAAGAGCTGCTACGTGAAC
p53 methylated DNA	Forward: ATCGTTATTCGGTTTGTTTTC Reverse: CGAACACGACTCCCAGCTAA
p53 unmethylated DNA	Forward: ATCGTTATTCGGTTTGTTTTC Reverse: CGAACACGACTCCCAACTAA
P53 mRNA	Forward: GGCTTCCACCTGGGCTTCCTGCAG Reverse: CCTCATTACAGCTCCCGGAACATCTC
E-cadherin methylated DNA	Forward: GGTTTAATTTCGGTTTGTTCGATCGTATTC Reverse: GAACTCCCATAACGAACCCG
E-cadherin unmethylated DNA	Forward: GTTTGGTTTAATTTGGTTTGTGTTGATTGTATTTG Reverse: ACCAAACTCCCATAACAAACCCA
E-cadherin mRNA	Forward: AAGTGACCGATGATGATGCCA Reverse: CTTCTCTGTCCATCTCAGCG
GAPDH mRNA	Forward: TCCATGACAACTTTGGCATTG Reverse: TCACGCCACAGCTTTCCA

modified with an EZ DNA Methylation kit (ZYMO Research Co, Orange, CA, USA) according to the manufacturer's instructions. Briefly, 1.0 µg denatured DNA was treated with sodium bisulfite (50° C, approximately 16 hours) in the dark. Samples were then applied to columns and desulfonation was conducted. DNA was subsequently eluted with supplied elution buffer. Generally, 1.0 µL of bisulfite-converted DNA solution was used in subsequent methylation-specific polymerase chain reactions [10].

Methylation-specific Polymerase Chain Reaction

Methylation-specific polymerase chain reaction (MSP) of tumor suppressor genes including P16, P53, and E-cadherin was conducted with the EpiTect MSP Kit (Qiagen) according to the manufacturer's instructions (Table 1). Polymerase chain reaction products were loaded in 1.5% agarose gels and visualized using ethidium bromide.

Semiquantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from cells using the RNeasy plus mini kit (Qiagen) and cDNA was generated using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). The primer sequences for reverse transcription-polymerase

chain reaction (RT-PCR) are listed in Table 1. The cycling parameters used for all reactions were as follows: 94° C for 5 minutes; 30 cycles of the following: denature for 45 seconds at 95° C, annealing for 30 seconds (53–56° C), and extend for 45 seconds at 72° C. RT-PCR was performed using a Bio-Rad MyiQ thermal cycler (Bio-Rad). GAPDH served as a control gene, and the expression of target genes was normalized to the expression of GAPDH. Gradient dilution (1:1, 1:2, and 1:4) of RNA samples from different cell groups was compared, respectively, to verify the quantitative difference of gene expression. RT-PCR analysis was performed using ImageJ software (Version 1.32j; National Institutes of Health, Bethesda, MD, USA) where the integrated density (product of the area and the mean gray value) of bands was calculated. All molecular bands were normalized to GAPDH.

Cell Proliferation Assay

K7M2 cells were plated at 1000 cells per well in a 12-well plate and cultured in PM with or without 4% CEE. Images of cells were taken Day 0 and Day 4 of culturing. The approximate population doubling time (PDT) as determined as follows: $2^n = \text{cell number at harvest time} / \text{cell number initially plated}$; "n" refers to the number of doublings during the period of cell culture (4 days = 96 hours); thus, $\text{PDT} = 96 \text{ hrs}/n$.

Cell Survival Assay After Exposure to Oxidative Stress

K7M2 cells cultured in 12-well plastic plates were incubated with or without 4% CEE for 4 days, and the antioxidant capacity was assessed by exposure to oxidative stress (250 μ M H₂O₂ in PM) for 6 hours. Propidium iodide (PI) was added to the medium (1 μ g/mL) and apoptotic cells were identified with positive PI staining.

Cell In Vitro Invasion Assay

In vitro invasion capacity of K7M2 cells with or without CEE treatment was assessed using a real-time cell invasion and migration assay system (ACEA Biosciences, Inc, San Diego, CA, USA) with a 16-well transwell plate (CIM-plate 16; Roche Diagnostics GmbH, Mannheim, Germany). The surface of the wells in the upper chamber was coated with 2.5 Matrigel (BD BioSciences, Bedford, MA, USA). Serum-containing medium (10% FBS) was added to the wells of the lower chamber. Two $\times 10^4$ cells in 100 μ L of serum-free medium were seeded in the upper chamber of each well. The migration of the cells through the Matrigel was monitored by the system every 15 minutes for 24 hours. Data analysis was carried out using RTCA Software 1.2 (Roche Diagnostics, Mannheim, Germany) supplied with the instrument.

Actin Staining

Organization of actin in osteosarcoma cells was assessed using the phalloidin conjugated with Alexa Fluor 488 (Invitrogen, Grand Island, NY, USA). Cells were washed twice with phosphate-buffered saline (PBS), fixed in 3.7% formaldehyde solution for 10 minutes at room temperature, and washed two more times with PBS. The cells were then permeabilized in 0.1% Triton X-100 for 20 minutes and washed again with PBS. For each well, a staining solution of 5 μ L of methanolic stock solution phalloidin with 200 μ L PBS and 1% bovine serum albumin was added. The staining solution was kept in the wells for 20 minutes, and then the wells were washed again with PBS. This made the actin appear green under a fluorescence microscope.

Statistical Analysis

At least three samples obtained from each subject were pooled for statistical analysis of all results from this study, and the results are expressed as mean $\pm$ SD. The differences between two means (untreated samples versus samples treated with 2% or 4% CEE treatment) were considered to be statistically significant if *p* value was < 0.05 . A *t*-test (Microsoft Office Excel; Microsoft Inc, Redmond, WA,

USA; paired two samples for means) was used to determine statistically significant differences between two means.

Results

Highly Metastatic K7M2 Cells Display Greater DNA Methylation of Tumor Suppressor Genes Compared With Less Metastatic K12 Cells

Compared with less metastatic K12 cells, highly metastatic K7M2 cells feature greater DNA methylation of these three tumor suppressor genes (Fig. 1A). Also, conventional polymerase chain reaction analyses showed that the mRNA level of these genes was significantly lower in K7M2 cells (Fig. 1A), which is expected as a direct result of epigenetic silencing caused by DNA methylation.

Chick Embryo Extract Promotes the Demethylation of Tumor Suppressor Genes (ie, P16, P53, and E-cadherin) in K7M2 Cells and Activates Their Expression

To determine if CEE changed the methylation status of tumor suppressor genes in highly metastatic osteosarcoma cells, MSP analysis was conducted with K7M2 cells after treatment with different concentrations of CEE (0%, 2%, or 4%). Our results demonstrated a concentration-dependent effect of CEE that reversed the DNA methylation of p16, p53, and E-cadherin genes (Fig. 1B). Also, conventional polymerase chain reaction analyses showed that the mRNA level of these genes was upregulated with CEE treatment (Fig. 1B), which is expected as a direct result of DNA demethylation and the release of gene silencing. In addition, changes on the protein level of the genes, specifically E-cadherin and p53, was analyzed by immunocytochemistry staining of the cells. Results showed that increased deposition of both E-cadherin and p53 protein was increased by 4% CEE treatment (Fig. 1C).

Chick Embryo Extract Represses Proliferation and Triggers Morphological Changes Involving Altered Interaction of E-cadherin and F-actin

Proliferation assays were conducted to discover the effect of CEE on the proliferation of K7M2 and K12 cells. The proliferation of K7M2 cells appeared to be statistically inhibited during 4 days of CEE treatment (4%) (Fig. 2A). However, little effect on the proliferation of K12 cells was found with CEE treatment. Also, after 4 days of CEE treatment (4%), morphologic changes of K7M2 cells occurred, which produced cellular morphology reminiscent of the less metastatic K12 cells (Fig. 2C).

Fig. 1A–C DNA methylation status of K7M2 and K12 cells and effect of CEE treatment of K7M2 cells. **(A)** With MSP, more methylated DNA (less unmethylated DNA) of p16, p53, and E-cadherin genes was observed in K7M2 cells. With regular PCR, a lower level of mRNA of these tumor suppressor genes was observed in K7M2 cells. **(B)** Four days of CEE treatment of K7M2 cells caused a concentration-dependent decrease of methylated DNA of p16, p53, and E-cadherin genes and increase of both unmethylated DNA and mRNA. **(C)** By immunocytochemistry staining of the cells, increased protein level of E-cadherin and p53 were demonstrated with 4% CEE treatment.

K7M2 cells had more abundant cytoplasmic projections, whereas K12 cells and CEE-treated K7M2 cells were more smooth and polygonal. Furthermore, the morphologic change of K7M2 cells was accompanied with an alteration in distribution pattern of F-actin (Fig. 2D), indicating a change in the cytoskeletal structure of these cells. Costaining of E-cadherin and F-actin in the cells further revealed increased colocalization of the two proteins on the cell surface with 4% CEE treatment compared with nontreated control cells (Fig. 3). This observation may indicate the increase of E-cadherin on the cell surface at cell-cell or cell-ECM adhesion sites to which the actin cytoskeleton is anchored.

Chick Embryo Extract Decreases the Oxidative Stress Resistance and the In Vitro Invasion Capacity of K7M2 Cells

Our previous study has demonstrated strong oxidative stress resistance of K7M2 cells [31]. PI staining of K7M2 cells incubated with hydrogen peroxide (H_2O_2) displayed significantly increased percentages of apoptotic cells (PI-positive) in K7M2 cells after 2 days of CEE

treatment compared with cells without CEE treatment (Fig. 4A). We hypothesized that the epigenetic reactivation of tumor suppressor genes and changes in cell morphology of K7M2 cells with CEE treatment would result in changes of in vitro cell invasiveness. The invasion capacity of K7M2 cells with or without CEE treatment in 2.5% Matrigel was assessed using a real-time cell invasion and migration assay system. Significantly diminished invasion capacity was observed in K7M2 cells after 4 days of CEE treatment (Fig. 4B).

Discussion

Osteosarcoma is the most common primary malignancy of bone. Despite aggressive treatment, 5-year survival is only 65% to 70% and has remained at this level for over two decades. Current treatment strategies fail patients who are diagnosed with pulmonary metastatic disease or develop it during the course of their treatment. Novel, biologically intelligent, antimetastatic treatment options are therefore required to improve the prognoses of patients with osteosarcoma.

Fig. 2A–D CEE represses proliferation and change morphology of K7M2 cells. **(A)** Proliferation assay showed much less number of K7M2 cell after 4 days of incubation with CEE compared with nontreated K7M2 cells. **(B)** Proliferation rate of K7M2 cells with different concentrations of CEE treatment (0%, 2%, and 4%) as a

function of time is shown. **(C)** Cell morphology of K7M2 cells was changed to be similar to that of K12 cells after 4 days of incubation with CEE (4%). **(D)** Stain of actin demonstrated change of cytoskeleton structure. **(E)** Actin staining demonstrated alterations in cytoskeletal structure.

To this end, we have investigated the possibility that epigenetic modulation with CEE might alter the metastatic potential of highly metastatic osteosarcoma cells in vitro. There are several limitations to this study. First, this experiment was performed entirely in vitro using two cell lines. Future studies will attempt to discern what the “magic molecule(s)” within CEE might be responsible for the effects we have described. Additionally, considerable effort will be required to design a method by which we might translate these in vitro data into a feasible in vivo strategy to diminish the metastatic biology of osteosarcoma within a tumor microenvironment.

DNA methylation status of tumor suppressor genes p16, p53, and E-cadherin in cultured K7M2 and K12 cells, MSP analyses were conducted based on previous literature implicating these genes in osteosarcoma biology [10]. P16 expression has been shown to be altered in human and rat osteosarcoma, which involves DNA methylation at its promoter site [4, 21, 36]. Also, methylation of the p16 promoter was found to be associated with a malignant phenotype in

some tumor cells [26]. P53 is also involved in osteosarcoma tumorigenesis [12, 18], and epigenetic alterations of this gene contribute to the pathogenesis of osteosarcoma [35]. E-cadherin performs a key role in cell adhesion, and loss of E-cadherin is known as a key step leading to tumor metastasis [3, 31, 32]. Negative expression of E-cadherin was found to be correlated with higher metastasis and a shorter overall survival in human patients with osteosarcoma [24]. The methylation status of these three tumor suppressor genes was therefore investigated in this study.

Our results suggest that osteosarcoma cells with higher metastatic potential feature more DNA methylation of the aforementioned tumor suppressor genes, suggesting that DNA demethylation may serve to suppress osteosarcoma metastases. To our knowledge, our study is the first to investigate the application of CEE as a method by which to study tumor reversion through epigenetic reprogramming. We have demonstrated that CEE is effective in altering the DNA methylation status of tumor suppressor genes in highly metastatic osteosarcoma cells.

Fig. 3 CEE treatment increased colocalization of E-cadherin and F-actin. Costaining of E-cadherin and F-actin in the cells showed increased colocalization of the two proteins on the cell surface with 4% CEE treatment compared with nontreated control cells.

Fig. 4A–B CEE decreases antioxidative stress capacity and in vitro invasion capacity of K7M2 cells. **(A)** Two days of CEE (4%) treatment resulted in a higher ratio of PI + cells when cells were incubated with hydrogen peroxide (250 μ M). **(B)** Two days of CEE (4%) treatment resulted in decreased in vitro invasion capacity in 2.5% Matrigel.

The involvement of epigenetic dysregulation in the development of various tumors, including osteosarcoma, has been reported. Recent studies on the epigenetic reprogramming of cancer cells have revealed the potent effects of embryonic factors on the reversal of metastatic phenotypes [6, 34]. These include studies with amphibian oocyte extracts [1] or zebrafish embryo extracts [8]. Epigenetic reprogramming of metastatic melanoma cells had been previously reported when the cells are injected into chick embryos [11, 27]. Here we have shown for the first time the application of extracts from chick embryos in epigenetic reprogramming of osteosarcoma cells.

CEE has been derived from 9- to 12-day-old chick embryos [33]. CEE has been previously used as an undefined medium component for the cultivation of various stem cells [16, 23, 33] despite the fact that the mechanism behind its capacity to maintain and expand stem cells was not known. Our current results suggest that DNA demethylation may serve to repress osteosarcoma metastases. They demonstrate that CEE may promote the transformation of highly metastatic osteosarcoma cells to less metastatic cells. This study may present a new method to investigate osteosarcoma tumor reversion by epigenetic reprogramming.

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