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Effect of selenocystine on gene expression profiles in human keloid fibroblasts

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ABSTRACT

In this study, selenocystine, a nutritionally available selenoamino acid, was identified for the first time as a novel agent with anti proliferative activity on human keloids.
The 20 μM concentration after 48 h treatment used here was the most effective to reduce keloid fibroblast growth. We analyzed the gene expression profile of selenocystine treatment response in keloid fibroblasts by the microarray system to characterize the effects of selenocystine on human keloids.
The major alterations in keloid fibroblasts following selenocystine exposure included up-regulation of the genes encoding cell death and transcription factors. Prominent down-regulation of genes involved in development, cell adhesion and cytoskeleton, as well as extra cellular matrix genes, usually strongly up-regulated in keloids, resulted following selenocystine exposure.
The range of the down-regulated genes and the degree of the decreased expression appeared to be correlated with the degree of the morphological alterations in selenocystine treated keloids.

1. Introduction

Keloids (OMIM 148100) are benign skin tumors occurring during wound healing in genetically predisposed patients. Keloids are characterized by the proliferation of dermal fibroblasts, overproduction of extracellular matrix components (ECM), an increased infiltration of inflammatory cells including lymphocytes, mast cells (MCs) and macrophages. Keloids spread to invade normal skin beyond the boundaries of the original wound and do not regress spontaneously [1].

Worldwide keloid prevalence varies by geographic ancestry from 0.09% in Great Britain to 16% in the Congo [2]. Although keloid prevalence in the United States is not well documented, they are fairly common in young women and African Americans [3]. Keloids tend to be present, on the face (with cheek and earlobes predominating), upper extremities, chest, presternal area, neck, back, lower extremities and breasts.

Previously, we found that in keloid fibroblasts a p53 (TP53) under expression, due to the sequence mutations, in concert with ΔNp63 (an isoform of the TP63 gene) activation, is central in the mechanism involved in keloid proliferation. Suppression of apoptosis might contribute to keloid development by means of accumulation of continuously proliferating cells whereas the disruption or elimination of genetically altered cells might decrease tumor potential [4–6]. The exact etiology is still unknown and until today, there is no appropriate treatment yet for keloid disease. It is reasonable to believe that the lack of effective therapies is due to an insufficient understanding of the disease pathology and the lack of an animal model.

Epidemiological studies, preclinical investigations and clinical intervention trials support the role of seleno-compounds as potent cancer chemo-preventive agents [7] especially for several major cancers, including prostate, lung, colon and liver cancers [8,9].

Growing evidence suggested that reactive oxygen species (ROS) played an important role in cancer cell apoptosis induced by many anticancer agents. Interestingly, ROS production has been linked to cytotoxicity of selenum compounds [10,11]. Selenocystine, a nutritionally available seleno-amino acid, was identified as a novel agent with broad-spectrum antitumor activity. Several human cancer cell lines were shown to be susceptible to selenocystine, inducing a dose-dependent apoptosis in A375, HepG2 and MCF7 cells. Mechanistic studies showed time- and dose-dependent increases in intracellular reactive oxygen species (ROS) in susceptible cancer cells (MCF7 and HepG2 cells) treated with selenocystine, that was not observed in non-susceptible normal human fibroblast Hs68 cells. ROS has been shown to play a key role in the signaling pathway of selenocystine-induced, DNA damage-mediated apoptosis in susceptible cancer cells [12,13].

Despite its potential applications in pharmacology and cancer therapy, limited information is available on the action mechanism of selenocystine.

Since we previously found a significant correlation between ROS generation and apoptosis data, we might hypothesize that ROS could be an upstream mediator for p53 activation in keloid fibroblasts, leading to p53 activation that failed in keloids due to p53 mutations together with Δp63 over-expression [4–6].

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In this research, we investigated, for the first time, if selenocystine has an anti-proliferative effect on keloid fibroblasts, and we studied the transcriptional response following exposure to selenocystine on the gene transcription level in keloids by the use of an array gene chip. Functional genomic approaches have become a new strategy for biological studies, generally by use of DNA microarray systems. Such DNA microarray technologies can be used to monitor unclear gene interactions in a global way in human cells in response to therapeutical treatment.

Our results demonstrated an in vitro anti-proliferative activity of selenocystine in keloid fibroblasts, decreasing keloid tumor potential. Moreover, the DNA microarray system evaluated the differential gene expression patterns of keloid fibroblasts in response to treatment with selenocystine, showing that involvement of several genes, especially those belonging to the cytoskeleton and collagen families are likely to be responsible for susceptibility to selenocystine effects in keloid fibroblasts. Keloids are characterized by an abnormal composition of Extra Cellular Matrix proteins, which include elevated levels of collagen proteins [13]; these data are consistent with the fact that keloid lesions are extremely abundant; in fact it is reported that many genes, encoding general extracellular matrix proteins such as the collagen family, are strongly up-regulated in keloid lesions [14].

Our results demonstrated that DNA microarray system can be employed to evaluate the differential gene expression patterns of keloid fibroblasts in response to selenocystine exposure to a better understanding of the genes involved in the treatment which may potentially lead to the development of effective therapeutic treatments.

2. Materials and methods

2.1. Cell cultures and treatment

Fresh tissues were obtained from skin lesions of 16 patients with keloids for establishing the primary cell cultures. Fibroblasts from keloids were isolated from samples following the same method established by Lim et al. [15]. All experiments used cells between the second and third passage. No morphological and biochemical differences were found with the passage. Fibroblast cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 10 μg/mL streptomycin, and 50 IU/mL penicillin in 5% carbon dioxide at 37 °C.

From the second passage treatment time course experiments were set up for 24, 48, and 72 h without serum starvation, adding different concentrations of selenocystine (10, 20, 40 μM respectively) dissolved in 0.5 N HCl.

2.2. Phase contrast microscopy

Phase contrast images of control and treated cells were generated with the aid of a Zeiss Axiovert 40 CFL inverted microscope equipped with a Canon Power Shot G6 digital camera.

2.3. Cell viability analysis

To assess the cell metabolic activity, the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was carried out using a modified method described by Li et al. [16]. Briefly, 5×10^4 cells were seeded in each well containing 100 μl RPMI-1640 medium supplemented with 10% fetal bovine serum in a 96-well plate. After incubation for 24, 48 h and 72 h respectively 50 μl MTT (Sigma-Aldrich, St. Louis, USA) (5 mg/ml stock solution) was added and incubated at 37 °C for another 4 h. Then 0.2 ml of DMSO was added to stop the reactions. The absorbance of each well was determined spectrophotometrically at 490 nm by a microplate reader (Bio-Tek, Rockville, USA). All results shown are mean ± SD of at least three separate experiments, measuring each parameter by triplicate ($n=3$). Statistical significant differences were tested by one way analysis of variance (ANOVA), and, when the F value was significant, by Student–Newman–Keul's test. P value less than 0.05 (*) was considered statistically significant.

2.4. RNA isolation

Total RNA of fibroblasts from untreated keloids and treated keloids was extracted with NucleoSpin RNA II (Macherey-Nagel) according to the manufacturer's instructions. A DNase I treatment was applied to remove traces of contaminating DNA. Good-quality messenger RNA is an essential prerequisite for the high throughput messenger RNA profiling; care was taken to exclude cell cultures with degraded RNA or poor quality. RNA quality and quantity was checked for all the samples before proceeding to all the analysis. Quality of total RNA was checked using Agilent 2100 Bioanalyzer platform (Agilent Technologies).

2.5. Microarray processing and data extraction

1 μg of each total RNA sample was used. To produce Cy3-labeled cRNA, the RNA samples were amplified and labeled using the Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies) following the manufacturer's protocol.

Yields of cRNA and dye-incorporation rate were measured with ND-1000 Spectrophotometer (NanoDrop Technologies). The hybridization procedure was performed using the Agilent Gene Expression Hybridization kit. Briefly, 1.65 μg of Cy3-labeled fragmented cRNA in hybridization buffer was hybridized overnight to Whole Human Genome Oligo Microarrays 4x44K (Agilent Technologies).

Microarray hybridization and washing were performed using reagents and instruments (hybridization chambers and rotating oven) as indicated by the manufacturer (Agilent Technologies). Microarrays were scanned using Microarray Scanner System (Agilent Technologies).

2.6. Microarray data analysis

The 20 CEL files generated by the Agilent Microarray Suite version 5.0 (MAS 5.0; Agilent) were converted into DCP files using dChip V1.3 software (for more information, access the following Web site: http://www.dchip.org).

The Agilent Features Extraction Software (FES) was used to read out and process the microarray image files. FES output data files were further analyzed using the Rosetta Resolver gene expression data analysis system (Rosetta Biosoftware).

The DCP files were normalized, and raw gene expression data were generated using model-based analysis of the dChip system.20. For comparison of global gene expression profiles between two sample sets (e.g., control vs CJD samples), Significance Analysis of Microarray (SAM) software (for more information, access the following Web site: http://www.stat.stanford.edu/_tibs/SAM/index.html) was used with two-class response analysis. SAM is a statistical method adapted specifically for multiple comparison issues in the context of microarray data, and it has been shown to be more accurate than the calculation of fold change.21 SAM identified genes with statistically significant changes in expression by assimilating a set of gene-specific t tests. Each gene, i, is assigned a score, $d(i)$, representing the relative difference of this gene. The $d(i)$ score is generated based on the ratio of the change in expression of gene i in different states (e.g, in control cases and patients) relative to the standard deviation of repeated measurements. SAM also estimates the percentage of genes identified by chance, based on permutations of the measurements for each gene. In this study, genes were considered differentially expressed if they changed more than 2-fold (see Results) with a $p<0.01$. For hierarchical clustering, gene expression data generated by dChip software were log transformed, median centered, and analyzed for clustering according to Eisen and colleagues [17].