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DNAJA4 deficiency enhances NF-kappa B-related growth arrest induced by hyperthermia in human keratinocytes

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ABSTRACT

Background: Hyperthermia is an effective treatment against cancer and human papillomavirus (HPV) infection. Previous studies have shown that heat shock proteins are crucial to the action of hyperthermia.
Objectives: To examine the effects of hyperthermia in combination with DNAJA4-deficiency on human keratinocytes and Condyloma acumunatum (CA) tissues.
Methods: HaCaT cells were subjected to 44 °C (compared to 37 °C) waterbath for 30 min for stimulation. Foreskin or CA tissues obtained from patients undergoing circumcision or pathological examination were bisected and subjected to similar treatments. DNAJA4-knockout (KO) HaCaT cells were generated with CRISPR/Cas9 technology. mRNA and protein expressions were determined using rt-qPCR and western-blotting. Cell cycle distribution, apoptosis and senescence were analyzed by flow cytometry.
Results: DNAJA4 was induced in HaCaT cells, foreskin and CA tissues subjected to hyperthermia at both transcriptional and translational levels. NF-kB,[3] was activated by hyperthermia in HaCaT cells, and further enhanced by DNAJA4-deficiency. Transcription of TNF-α[4]; IL-1B,[5] TNFAIP3[6] and IL-8[7] were induced in HaCaT cells subjected to hyperthermia. DNAJA4-knockout promoted transcriptions of TNF-α and IL-1B, whereas decreased that of TNFAIP3 and IL-8. Reduced cell survival, proliferation and viability were demonstrated using flow cytometry and MTS assays. Furthermore, NF-kB inhibitors reversed most of the phenotypes observed.
Conclusions: Hyperthermia reduced HaCaT cell proliferation and promoted cytokine expressions responsible for anti-viral activity, mainly through a NF-kB dependent pathway. DNAJA4-deficiency enhanced the activation of NF-kB by hyperthermia in HaCaT cells, indicating that DNAJA4 may be a promising therapeutic target for use in the treatment of cutaneous HPV infections.

1. Introduction

Exogeneous hyperthermia, defined as a 30–60 min exposure to a thermal stimulus of 40–44 °C, has been employed in treatment of an array of diseases [1]. This technique has proved effective in the treatment of various of cancers such as cervical, bladder and head and neck cancer [2–4]. Within our clinic we have used local hyperthermia at 44 ± 0.1 °C in the treatment of plantar warts and found this procedure to be more effective than that of conventional therapies [5,6]. With this method, we have also successfully alleviated cutaneous warts in patients with diabetes mellitus [7], systemic lupus erythematosus (SLE) [8], Darier disease [9] and pregnancy [10], all of which had experienced failures with conventional treatments.

Heat shock proteins (HSPs) are acute phase reaction proteins (ARPs) which are highly conserved among spieces and show

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[3] NF-kB: nuclear factor-kappa B.
[4] TNF-α: tumor necrosis factor-alpha.
[5] IL-1B: interleukin-1 beta.
[6] TNFAIP3: Tumor necrosis factor alpha-induced protein 3.
[7] IL-8: interleukin-8.

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increased expression during stressful conditions including hyperthermia. The most well-studied member of the HSP family, HSP70s, serves as a molecular chaperone which functions against protein misfolding and aggregation and protects cells from injuries under stressful conditions such as heat [11] and UVB irradiation [12], whereas HSP90s are broadly associated with factors that foster tumorigenesis [13]. Inhibitors that target HSP90s have been used as therapeutic agents in the treatment of cancer [14]. Among all identified HSPs, DnaJ/HSP40s, with molecular weights around 40 kDa, function as co-chaperones of HSP70s [15] and facilitate HSP90s protective ability [16]. As a member of the HSP40s family, DNAJA4, has received little attention and its specific functions remain to be determined.

Nuclear factor-kappa B (NF-kB) is one of the most important transcription factors which translocates to the nucleus on activation and initiates multiple gene transcriptions responsible for immunological activity and inflammation *in vivo* [17]. NF-kB also affects cell proliferation, however, its influence on cell proliferation seems to be tissue or cell type-specific. For example, while enhanced expression of NF-kB promotes proliferation of several cancer cells [18] and inhibition of NF-kB shows anti-proliferative activity [19], NF-kB activation is responsible for growth arrest in stratified epithelium [20] and inhibition of NF-kB via the dominant negative IkBa factor promotes tumorigenesis in human squamous-cell carcinomas [21]. NF-kB is also a heat-shock responsive protein that reacts to hyperthermal conditions [22] and up-regulation of HSP70 after heat shock pre-treatment alters NF-kB activation [23,24]. These findings indicate that hyperthermia-induced heat shock proteins may be one of the regulators of cellular proliferation, immune responses and inflammation via the NF-kB pathway.

In this study, we examined one specific HSP40 (DNAJA4), which is induced by hyperthermia in human keratinocytes. Specifically, the goal of this report was to assess the influence of DNAJA4 on the NF-kB signaling pathway and cell proliferation in response to hyperthermia.

2. Materials and methods

2.1. Cell culture and hyperthermia treatment

HaCaT cells were purchased from Kaiji Jiangsu, China and cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (Biological Industries, Israel) containing 1% penicillin and streptomycin (Biological Industries, Israel). Cells were incubated at 37 °C in a humidified incubator with an atmosphere of 5% CO_2. HaCaT cells were digested by adding Trypsin EDTA Solution A (0.25% trypsin with 0.02% EDTA; Biological Industries, Israel) for subculturing. For hyperthermia stimulation, two million HaCaT cells were counted with use of a Scepter™ Handheld Automated Cell Counter (Merck Millipore, Germany), seeded in 100 mm Petri-dishes and were grown overnight to achieve 60–70% confluency. The dishes were then carefully immersed in a 44 °C (±0.1 °C) water bath (Thermo Fisher Scientific, USA) for 30 min. After hyperthermia treatment, cells were returned to 37 °C for recovery and harvested at varying recovery time points as described below. Control groups were treated likewise with 37 °C water bath.

2.2. Tissue and hyperthermia treatment

Fresh foreskin tissues were obtained from patients undergoing circumcision in the Urology Department of the No.1 hospital of China Medical University. Residual fresh Condyloma acuminatum (CA) tissues were obtained from patients undergoing pathological examination or surgical treatment in the Dermatovenerology Department of the No.1 hospital of China Medical University. Fresh tissue samples were immediately bisected and separately placed in two culture dishes with DMEM medium.While one half of tissue was immersed in a 44 °C (±0.1 °C) water bath (Thermo Fisher Scientific, USA) for 30 min, the other half was treated likewise in a 37 °C (±0.1 °C) water bath. After hyperthermia, tissues were returned to 37 °C within an incubator for 8 h before being fixed with formalin.

2.3. Lentivirus transfection

HaCaT cells were carefully seeded in 100 mm Petri-dishes and cultivated in an antibiotic-free medium before lentivirus transfection. DNAJA4-gRNA-EGFP lentivirus (KO group), Negative Control-gRNA-EGFP (NC group) and Cas9-puro lentivirus (Genechem, Shanghai, China) were transfected into HaCaT cells in transfection reagent Polybrene (Genechem, Shanghai, China) and Eni.S. (Genechem, Shanghai, China). Puromycin (Sigma Aldrich, USA) at 1.0 ug/ml was used to screen for HaCaT cells that were successfully transfected. Three specific gRNAs, as designed based on the DNAJA4 sequence (NM_018602), were as follows:

+ TTATGACCAAGGCGGAGAGC
+ TAATGGAGTCACGAAGAAAT
− TAATTGCCTGCTCTCCGCCT(antisense)

2.4. Inhibition of NF-kB activity

NF-kB specific inhibitor PDTC (Sigma Aldrich, USA) and Helenalin (ab146197, abcam, UK) were prepared at a concentration of 100 mM (PDTC, dissolved in water) or 100 mM (Helenalin, dissolved in DMSO) and separately added to culture medium to reach a final concentration of 20 μM or 10 μM respectively 1 h prior to hyperthermia treatment. Either of the inhibitors was kept remained in the culture medium during hyperthermia treatment and 37 °C recovery until further experiments. Inhibitor-negative groups were treated likewise with use of equal volume of respective solvent (water or DMSO) as compared to inhibitor-positive groups.

2.5. Real-time qPCR assay

In each experimental group, mRNA was extracted using the miRNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions and quantified with a ND1000 Nanometer. Complementary DNA (cDNA) was synthesized by utilizing 1 ug mRNA extracted as templates with the GoScript Reverse Transcription Kit (Promega) according to the protocols supplied.

Primer sequences for DNAJA4, IL-1B, P65(RELA), P50(NF-KB1), IL-8, TNF-α, TNFAIP3 and GAPDH were designed using Primer-Primier 6.0 software. The sequences were as follows,

DNAJA4 sense: TTATGACCAAGGCGGAGA
DNAJA4 antisense: CCACCAACACCTTCACAT
IL–1 B sense: GGCTTATTACAGTGGCAATG
IL–1 B antisense: GTAGTGGTGGTCGGAGAT
P65 sense: CTGCGTGTTGAAAGATGATA
P65 antisense: CTTGAGAGGTGCTGATGTA
P50 sense: TCCACCTTCATTCTCAACTT
P50 antisense: CACCACATCTTCCTGCTTA
IL-8 sense: AAGGTGCAGTTTTGCCAAGG
IL-8 antisense: CAACCCTCTGCACCCAGTTT
TNF-α sense: TCTTCTCGAACCCCGAGTGA
TNF-α antisense: ATGAGGTACAGGCCCTCTGA
TNFAIP3 sense: TCAGGACACAGACTTGGTA
TNFAIP3 antisense: CAGTTCCGAGTATCATAGCA
GAPDH sense: AAGAGCACAAGAGGAAGAGAGAGAC
GAPDH antisense: GTCTACATGGCAACTGTGAGGAG

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