ABSTRACT

Objectives: The expression levels of heat shock proteins (HSPs) are elevated in many cancers, and this overexpression is often associated with both a poor survival and a therapeutic outcome. Curcumin is an anti-cancer agent that also induces a heat shock response. HSPs confer resistance to curcumin-induced apoptosis in cancerous cells. The aim of the current study was to analyze variations in the expression levels of hsp gene in response to polymeric-nanocurcumin (DNC) to determine whether these alterations have inhibitory effects on apoptosis or promote it. Methods: The IC50 of DNC on AGS cell line was determined by the MTT assay. Following DNC treatment, the expression levels of hsp mRNAs were quantified by qPCR. Using siRNA, the Hsp27 gene was knocked down, and the DNC-induced apoptosis was determined by Annexin V test. Results: Three variants of Hsp27 and two members of Hsp40 and Hsp90 families were significantly up-regulated while one member of Hsp40 and Hsp70 families were down-regulated. As a result of Hsp27 knock down, the DNC-induced apoptosis was increased about 26% (26.19 vs 52.61). Conclusion: The overall effect of DNC on Hsp genes was induction, which suggests that DNC could cause stress in AGS cells. On the other hand, reduced levels of Hsp70-2 and DnaJC3 expression could be indicative of their involvement in DNC-induced apoptosis. In addition, as Hsp27 knock down led to an increase in apoptosis, it appears that Hsp27 confers resistance against DNC-induced apoptosis. Therefore, knock down of Hsp27 gene could be considered as a supplementary treatment beside DNC in cancer therapy.

Keywords: Heat shock proteins, Polymeric-nanocurcumin, Apoptosis, AGS cell line

INTRODUCTION

HSP proteins were first discovered by Ritossa in 1962 [1] and isolated by Tissieresin 1974 [2]. HSPs are highly conserved proteins in almost all organisms, induced in response to several environmental and physiological stresses [3]. HSPs help folding of proteins and prevent the aggregation of misfolded or unfolded proteins which would otherwise be inactivated. The expression levels of HSPs increase in response to several stress conditions such as hypoxia, heat shock, etc. [4]. In cancerous cells, the increase in HSP levels plays key roles in preventing spontaneous apoptosis happening in malignancies, and treatment induced apoptosis, the mechanism probably involved in tumor progression and resistance against therapies [5-8].

Curcumin is a polyphenolic compound [9] of turmeric plant (Curcuma longa) [10]. It has anti-cell proliferation, anti-mutation, anti-angiogenic and anti-oxidant properties [11-16]. Curcumin can also activate heat shock response [17] and by this mean it shows anti-invasive properties [18]. On the other hand, HSPs could confer resistance against curcumin induced apoptosis in cancerous cells [19].

One of the most prevalent human cancers and the second
cause of mortality among cancers worldwide is gastric cancer [20]. More than 90% of gastric cancers are of adenocarcinoma type. Mortality of this cancer is more than 50000 people per year worldwide [21]. This is a prevalent cancer in Iran especially in north and northwestern regions such as Golestan, Ardabil and Mazandaran provinces [22].

In the current study the AGS cell line originated from gastric adenocarcinoma, as a model system for gastric cancer, was treated with DNC. The expression levels of Hsps 27, 40, 70 and 90, which are important in gastric cancer, were analyzed by qPCR. It was observed that following DNC treatment, the expression levels of Hsp27, two variants of Hsp40 and two variants of Hsp90 were elevated while one variant of Hsp40 and Hsp70 were down-regulated.

MATERIALS AND METHODS

Cell culture: The AGS cell line was provided by the Pasteur Institute (Tehran, Iran). The cells were cultured in HamsF12 and RPMI culture medium (Gipco, Germany) in a 1:1 ratio supplemented with 10% FBS (Gipco, Germany), 100U/ml Penicilin and 0.1 mg ml⁻¹ streptomycin (Gipco, Germany) in 25 ml flasks. Cells were grown at 37 °C in a 5% CO₂ and 95% humidified atmosphere.

Cell viability and growth assay: In order to determine the IC₅₀ concentration of DNC, the MTT assay introduced by Mossmann [23] was performed. Briefly, cells were plated in 96 well plates and exposed to different concentrations of DNC, after a period of synchronization. After 24, 48 and 72h incubation periods, 20 µl of 0.5% (w/v) 3-(4,5-dimethylthiazol-2-ethyl) 2,5-diphenyltetrazolium bromide (MTT) in PBS was added to each well. Following four hours of incubation period, the water insoluble Formazan dye was solubilized by DMSO (200µl per well) and the optical densities were read by an ELISA reader (Bio Tek, U.S.A.). Preparation of DNC: The DNC solution was prepared according to Babaei et al., instruction [24].

DNC treatment: Cells were exposed to 13.5 µM of DNC in addition to their natural medium in 25 ml flasks for 24 h. siRNA transfected cells were also treated similarly, following six hours of transfection medium removal. An equal amount of PBS was added to the control cultured cells in place of polymerized nanocurcumin (PNC).

Primer Design

Primers were designed using AlleleID 6.1, and were checked by PrimerBLAST tools for specificity. The sequences of primers were as follows:

mRNA expression: RNA extraction was performed by Trizol reagents. First strand cDNA was prepared using PrimeScript™ RT reagent kit (Takara Bio, Japan) and amplified in ABI 7500 light cycler. The SYBR® Premix Ex Taq™ master mix was used for the amplification purposes. Efficiencies of amplification reactions were determined by Linreg 12.12. Real-time data were normalized related to Gapdh amplification, as an internal control. Data were analyzed according to the Soong model [25]. Statistical comparisons of data were performed with Student’s t-test, using GraphPad Prism, Version 5.00 and were considered significant if p < 0.05.

siRNA Treatment

siRNA was designed by Block-It RNAi Designers (https://rnaidesigner.invitrogen.com/maixpress) software and transfected into the cells by lipofectamin 2000 according to manufacturer’s recommendation (Invitrogen, Germany). The sequence of siRNA and its complementary strand was as follows:

Anti-sense strand: 5'-rGrUrCrArArGrArGrArUrCrArCrCrArUrCdTdT-3'
Sense strand: 3'-dTdTTrCrGrGrUrUrGrCrUrCrUrGrUrGrUrArG-5'

The scrambled sequence used as a control was 5'-GGUAUUCUCUCGGUACA-3'.

Annexin V test

Apoptotic cell death was confirmed by staining the cells with annexin V (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s instructions. Apoptosis was then monitored by flow cytometric analysis with a FACScan using Flowmaxsoftware (Partec Germany).

RESULTS

Determination of IC₅₀ Concentration of DNC

In order to determine the IC₅₀ concentration of DNC the MTT assay was performed for 24 and 72 h. To do this, the
Fig. 1. MTT assay. Graphs show the viability of AGC cells in the presence of different concentrations of DNC. The horizontal axis represents the drug concentrations and vertical axis stands for OD. A) 24 h of incubation period and B) 48 h. The IC_{50} of DNC was 13 µM for 24 h and 12 µM for 48 h.

Fig. 2. Quantitative RT-PCR on Hsp mRNAs. Nt stands for non-treated and t for treated with DNC. A) shows up-regulated and B) shows down-regulated Hsps.

Fig. 3. Hsp27 knock down by siRNA. Nt: non-treated cells, sc: cells treated with scramble, 24: cells treated with siRNA for 24 h, 48: cells treated with siRNA for 48 h and 72: cells treated with siRNA for 72 h.

AGS cells were exposed to different concentrations of DNC and after incubation period, the MTT solution was added. The IC$_{50}$ of DNC was 13 µM for a 24 h incubation period and 12 µM for 48 h (Fig. 1).

**Differential Expression Levels of hsp**

The differential expression of *hsp* mRNA levels was assessed by Real-time RT-PCR. Our data indicated that mRNA levels of *hsp* genes gradually respond to DNC treatment up to 18 h and then remain constant. Therefore, the mRNA expression was analyzed after 18 h of incubation with PNC. Overall, all Hsp27 variants were significantly upregulated in response to DNC treatment (*P* < 0.05). *DnaJC15* was downregulated while *DnaJC3* and *DnaJ/B1* were significantly upregulated (*P* < 0.05). The only Hsp70 variant responding to DNC treatment was *Hsp70A* which showed a reduced mRNA level. The Hsp90 variants AB1 and B1 were significantly upregulated (*P* < 0.05) while AA1 variant was not significantly altered (Fig. 2).

Following siRNA transfection, the *hsp* 27 was maximally repressed after 24 h and then gradually increased. Although after 72 h its mRNA levels still remained significantly lower than the untransfected cells (Fig. 3).

**Effects of Hsp27 Knock Down on DNC Induced Apoptosis**

In order to investigate the effects of Hsp27 knock down on DNC induced apoptosis, the Annexin V test was executed. These results revealed 26.42% more apoptosis in transfected cells compared to the untransfected cells (Fig. 4).

**DISCUSSIONS**

A great number of studies have been reported on the therapeutic application of curcumin, especially in cancer treatment, indicating specific apoptotic effects of curcumin on cancerous cells [26-28]. The mechanism of this specificity was the subject of many investigations. Among many other mechanisms, curcumin can lead to alterations in the expression levels of stress related proteins such as HSPs [17]. In the current study, an overall upregulation of *Hsps* mRNA expression were observed. This could be considered as an anti-inflammation and anti-cell proliferation response. From this point of view, HSPs compete with Raf-1 proteins to interact with Bag-1 protein [29]. In normal conditions, the interaction of Bag-1 with Raf-1 leads to the activation of downstream MEK and ERK and finally to cell proliferation [30]. The replacement of HSPs with Raf-1 could cease the DNA synthesis and therefore cell proliferation [31]. Moreover the induction of heat shock response could prevent the activation of NF-κB [32-35] executing anti-inflammation effects. On the other hands, curcumin could produce reactive oxygen species in cancerous cells [36] leading to apoptosis induction as a result of cellular stress. From this perspective, the induction of HSP proteins could be considered as a side effect of curcumin treatment and as a cell defense against the stress exerted by curcumin. In addition, it has been reported that curcumin could cause HSF-1 phosphorylation and nuclear translocation, leading to the activation of *hsp* genes [REFERENCE]. It is not clear that whether this overall induction of *hsp* has deleterious effects in cancerous cells or is the remnant of protective effects of curcumin at low concentrations. Nevertheless, it is obvious that in many types of cancers, the HSP levels are higher than normal [37] and this higher expression is associated with resistance to cancer therapy and poor prognosis [33,38,39].

Functionally, HSPs are stress responsive, but this does not mean that they are redundant. Different *hsp* responses to different stress conditions [37,40]. It is important therefore to determine which *hsp* respond to the given stress condition.

Hsp27 is a molecular chaperon, preventing apoptosis induced as a result of misfolded protein aggregation. Moreover, hsp27 could interact with apotosome and regulate it negatively. In addition, hsp27 prevents the release of cytochrome c from mitochondria and also interacts with procaspase 3, hampering apoptosis. The instability of cytoskeleton which takes place in response to stress conditions could lead to apoptosis. Hsp27, by stabilizing actin cytoskeleton, could prevent apoptosis [41]. It is therefore plausible that the induction of hsp27 observed in the current study, was due to cellular resistance against a curcumin induced stress. To test this hypothesis we knocked down the hsp27 gene by siRNA and then assessed the apoptosis induced by DNC. We observed that the apoptosis
induced by DNC was significantly increased in transfected cells compared to the untransfected ones (52.61% vs. 26.19%). Therefore, it seems that the upregulation of hsp27 in response to curcumin treatment could be considered as a cellular defense mechanism against stress conditions induced by curcumin.

The DnaJ family (hsp40) functions in protein folding, translocation, translation and degradation mostly via the activation of ATPase activity of hsp70 members [42]. The DnaJ1 variants, a type II member of hsp40 proteins, could participate in protein maturation and is required for the first step of an Hsp90 multi-chaperon complex formation with mutant p53 [43,44], implicating a potential role for DnaJ1 in the HSP90-mediated development and spread of cancer. The DnaJ3 protein was found both in the ER and cytoplasm of cells. In the ER, it selectively presents client unfolded or misfolded proteins to BiP, an ER member of hsp70 family, and stimulates BiP function while in the cytoplasm, DnaJ3 inhibits kinases PERK and PKR [45-48]. Activated PERK and PKR phosphorylate eIF2α, and inhibit cap dependent protein translation as a consequence [49]. This reduces nascent protein load of ER which is essential for cancerous cells to survive. Moreover, PERK itself could release NRF2 from its cytosolic inhibitor KEAP1 that leads to the expression of a number genes implicated in the antioxidant stress response. On the other hand, curcumin could induce oxidative stress in cancerous cells. Therefore, the upregulation of DnaJ3 could be considered as a pro-apoptotic effect of curcumin in the AGS cell line. The DnaJ15 gene, another hsp40 member which showed down-regulation in this study, is silenced by DNA methylation in early childhood brain tumors [50]. Low levels of this protein are also correlated with increased resistance to chemotherapy in ovarian cancer [51].

The HSP70 protein family is involved in misfolded protein repair and apoptosis induction. The mRNA levels of

![Fig. 4. The results of annexin V test. a: non-treated cells, b: treated with DNC, c: treated with DNC and siRNA, d: treated with scrambled RNA, e: treated with lipofectamine itself and f: treated with DNC and lipofectamine.](image)
HSPA1A, HSPA1B and HSPA1L did not show any significant alteration while HSPA2 mRNA level decreased significantly. The down-regulation of HSPA2 could lead to apoptosis induction in cancerous cells [52]. It has also been shown that the high expression level of HSPA2 is associated with metastatic properties of cancerous cells, while its inhibition significantly suppresses tumor growth [53]. Therefore, the effect of curcumin on the expression level of hspa2 could be regarded as an anti-cancer influence. The current study showed the induction of HSP90B1 and AB1 upon curcumin treatment which has protective effects against apoptosis.

In conclusion, as a result of DNC treatment of AGS cell line, the mRNA levels of hsp27 and 90 family members were increased, and this elevation may potentially correlate with cellular resistance against curcumin induced apoptosis. Knocking down of Hsp27 by siRNA increased the rate of apoptosis in response to DNC treatment, suggesting a potential therapeutic application of HSp27 inhibitors in combination with curcumin treatment. The alterations in DnaJB1 and DnaJC15 expression due to DNC treatment may be a cellular defense mechanism against curcumin induced stresses while the up-regulation of DnaJC3 following DNC treatment could have pro-apoptotic effects. The only hsp70 member that showed altered expression following DNC treatment was hspa2 which was down-regulated and possesses potential anti-metastatic and anti-cancer growth and progression effects. Overall, the majority of variations in the expression levels of Hsp genes in response to DNC could be regarded as a cellular defense mechanism against stresses exerted by this drug.

ACKNOWLEDGEMENTS

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**Table 1. The Sequences of Primers Used in this Study**

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