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Quercetin, apoptosis, heat shock

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Abstract

The present study was designed to investigate the correlation between the expression level of Hsp27 and Hsp72 and induction of apoptosis in HeLa cells in response to quercetin treatment. Treatment of HeLa cells with quercetin or with 1 hr period of hyperthermia (42°) increased the number of apoptotic cells. Inhibition of the expression of Hsp72 and Hsp27 in tumour cells by anti-sense oligonucleotides, enhanced the induction of apoptosis by quercetin. Heat shock itself had little effect on apoptotic cell death in these cells, but when combined with quercetin treatment, caused a significant increase in the number of apoptotic cells. These results suggest that the reduction of Hsps expression in the HeLa cell line promotes the induction of apoptosis by quercetin.

Keywords: Quercetin; Hsp27; Hsp72; Apoptosis; Anti-sense oligonucleotides

1. Introduction

All organisms respond to environmental and physiological stress at the molecular level, by the synthesis of a conservative group of proteins called heat shock proteins. These act as molecular chaperones, controlling the proper folding of newly synthesised polypeptides, the refolding of misfolded proteins and controlling translocation through cell membranes [1,2,10,20–23]. Genes encoding Hsps are also regulated by physiological processes like cell cycle, cell proliferation and differentiation [26]. Their expression can also be modulated by many conditions leading to apoptosis and which are associated with pathologic states like ischemia, fever, inflammation, infections and cancers [2,27], where the enhanced expression of Hsps has been reported for nearly all classes of tumours [3,7,8,24]. It is known that tumour cells are very resistant to cell death and the reason for that can be the ability of Hsps to protect cells from apoptosis [14,25]. This may explain the known fact that Hsps overexpression in tumour cells indicate poor prognosis and resistance to chemotherapy. Increased levels of Hsp27 have been detected in breast cancers, endometrial cancer and leukaemia, while elevated expression of members of Hsp70 family has been reported in high-grade malignant tumours. In breast cancers overexpression of Hsp70 is associated with short-term disease-free survival, metastasis and poor prognosis [3,7,8,19,24]. Thus, decreasing Hsps level in cancer cells would be beneficial. It seems that quercetin (3,3′,4′,5,7-pentahydroxyflavon), one of the most widely distributed bioflavonoids in the plant kingdom, which is present in most edible fruits and vegetables, exhibits such properties. It inhibits the growth of malignant cells through several mechanisms such as inhibition of glycolysis, enzyme synthesis; freezing the cell cycle and interacting with estrogen type II binding sites [4,9,17].

Our earlier experiments [29] and data of other authors [5] indicate that quercetin inhibits Hsp72 and Hsp27 expression in HeLa cells. The aim of this study was then to investigate the influence of quercetin and hyperthermia on HeLa cells survival under the conditions of blocked Hsp72 and Hsp27 expression by specific anti-sense oligonucleotides.

2. Materials and methods

2.1. Cells and culture conditions

In experiments, human negroid cervix carcinoma cell line (HeLa B, ECACC No 85060701) cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS) (v/v) was used. Cells at a density of 1×10^6 cells/mL

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were seeded on cover slides (for apoptosis detection) or in Falcon vessels (for heat shock proteins identification) and incubated at 37° in humidified atmosphere with 5% CO_2.

2.2. Cells transfection

For HeLa cells transient transfection, anti-sense oligonucleotides (Sigma) anti-Hsp72 (5′-CGCGGCTTT-GGCCAT-3′, according to [4]) and anti-Hsp27 (5′-TACT-GGCTCGCGGCG-3′, complementary to the initiation codon and four downstream codons of human gene Hsp27 [32]), conjugated with fluorescein at 5′ ends, were inserted into the cells using cationic lipid LIPOFECTIN® reagent (Gibco) [28].

Cells were seeded on the day before transfection in RPMI 1640 medium. For apoptosis detection, a sample containing 0.4 μg of one oligonucleotide and 10 μL of PLUS® reagent (Gibco) in 50 μL of serum-free medium, was mixed together and then 0.25 μL of LIPOFECTIN® reagent in 25 μL of serum-free medium was added. The sample was then incubated for 15 min at room temperature. During the incubation time, medium from above the cells was replaced by 200 μL of fresh serum-free medium. For the heat shock proteins detection, the amount of all reagents used was increased five times. After incubation, DNA-PLUS®–LIPOFECTIN® reagent complexes formed were overlaid on cell cultures and incubated at 37° in 5% CO_2 for 3 hr, then supplemented with 13% FBS, and incubated for next 24 hr. The level of transfection and the localisation of oligonucleotides in cells, were determined by hybridisation technique [30,31], using specific anti-fluorescein antibodies conjugated with alkaline phosphatase, and by fluorescent microscope analysis of fluorescein administration in the cells.

2.3. Heat and drug treatment

In our experiments, quercetin (Sigma) dissolved in dimethyl sulfoxide (DMSO) was used. The final concentration of DMSO in culture medium did not exceed 0.25%, which, as indicated in preliminary experiments, did not influence Hsp72 and Hsp27 expression. The cultures were preincubated with quercetin in concentration 15 μg/mL for 4 hr at 37°, heated at 42° for 1 hr, and transferred to 37° for 2.5 hr. To examine the influence of quercetin alone, HeLa cells were incubated in 37° for 7.5 hr only.

2.4. Single cell gel electrophoresis

DNA breaks, typical for apoptosis, were detected using the comet assay method. Nontransfected cells treated or not with quercetin and/or heat shock, were suspended in low melting point agarose in PBS at 37° and pipetted onto frosted glass microscope slides, precoated with a layer of normal melting point agarose. The agarose was allowed to set in 4° for 10 min and the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM Tris, 10% DMSO, pH 10) for 1 hr at 4°. Slides were moved to an electrophoresis tank with electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 12.5–13), kept in it for 30 min at 4°, before undergoing electrophoresis at 25 V for 25 min at the same temperature. Slides were then washed three times for 5 min with 0.4 M Tris–HCl, pH 7.5 and stained with acridine orange (2 μg/mL) overnight. Morphological analysis of nuclei with characteristic tails (comets) was performed under fluorescent microscope (Nikon E-800). At least 1000 cells in three independent repetitions of the experiments, in randomly selected microscopic fields, were counted and analysed statistically by χ^2 test at $P < 0.05$.

2.5. Immunoblotting

For Western blot analysis, cells were lysed in hot SDS-loading buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 10% glycerol, 100 mM DTT), boiled in water bath for 10 min, centrifuged at 10,000 *g* for 10 min and then the supernatant was collected. The protein concentration was determined by the Bradford method [16] and samples of supernatants containing exactly 80 μg of proteins were separated by 10% SDS–polyacrylamide gel electrophoresis [13]. Proteins were then transferred onto Immmobilon P membrane (Sigma). Following transfer, the membrane was blocked with 3% low fat milk in PBS for 1 hr, then incubated overnight with mouse monoclonal antibodies against Hsp27 (SPA-800, StressGen) and against Hsp72 (SPA-810, StressGen) diluted 1:1000. The membrane was washed three times for 10 min with PBS containing 0.05% Triton X-100 and incubated for 2 hr with a 1:30,000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma). The membrane was visualized with alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyphosphate (BCIP) and nitro-blue tetrazolium (NBT) in *N*,*N*-dimethylformamide (DMF). The experiment was repeated independently three times.

Quantitative heat shock protein levels were assessed using Bio-Profil Bio-1D Windows Application V.99.03 program. Significance levels were calculated using one-way ANOVA test. *P*-values lower than 0.05 were considered statistically significant.

3. Results

3.1. Induction of apoptosis by heat and quercetin treatment in nontransfected HeLa cells

To estimate the effect of heat shock and quercetin on the induction of apoptosis in HeLa cells, the comet assay technique was used. Incubation of cells for 7.5 hr in presence of quercetin at the concentration 15 μg/mL, increased the number of apoptotic cells by about 16%,

Table 1
Effect of quercetin and heat shock on the apoptosis induction in nontransfected HeLa cells, estimated by comet assay method

Conditions	Apoptotic cells (%)
C	0.95 ± 0.07
Q	15.65 ± 0.07*
H	2.15 ± 0.07*
QH	17.10 ± 0.14*

C: control of nontransfected cells; Q: cells incubated only with quercetin for 7.5 hr; H: cells exposed to heat shock at 42° for 1 hr; and QH: cells pretreated with quercetin (15 μg/mL) for 4 hr at 37°, then exposed to hyperthermia and incubated for 2.5 h at 37°.
* Values are significantly different from those of control at $P < 0.005$.

in comparison to control cells (Table 1). Above 17% of apoptoic death cells were noticed after additional exposure of quercetin treated cells to 42° for 1 hr. Heat shock itself had only a slight effect on apoptosis induction in the studied cell line.

3.2. The level of HeLa cells transfection by anti-sense oligonucleotides

To examine the effect of quercetin on HeLa cells with decreased Hsp72 or Hsp27 level, and to establish the direct involvement of these proteins in apoptosis, we decided to block Hsp72 and Hsp27 genes expression by transferring anti-sense oligomers anti-Hsp72 (5′-CGCGGCTTTGG-CCAT-3′) and anti-Hsp27 (5′-TACTGGCTCGCGGCG-3′) into the nuclei. The level of transfection was estimated by microscopic observation of the localisation of green light coming from fluorescein connected with anti-senses. Anti-sense oligonucleotides were detected mainly in nuclei (Table 2). In rest of the cells, the anti-senses were localised in cytoplasm or cells were not transfected at all.

Table 2
Localization of anti-sense oligonucleotides in HeLa cells estimated by hybridisation technique

Localization of oligonucleotides	Anti-Hsp27 (%)	Anti-Hsp72 (%)
Nucleus	60.80 ± 0.66	65.25 ± 4.36
Cytoplasm	24.20 ± 7.46	22.60 ± 2.74
Lack of oligonucleotides	13.80 ± 2.58	13.15 ± 4.01

3.3. Hsp27 and Hsp72 expression in transfected HeLa cells

HeLa cells transefected with anti-sense oligonucleotides anti-Hsp27 and anti-Hsp72 showed diminished expression

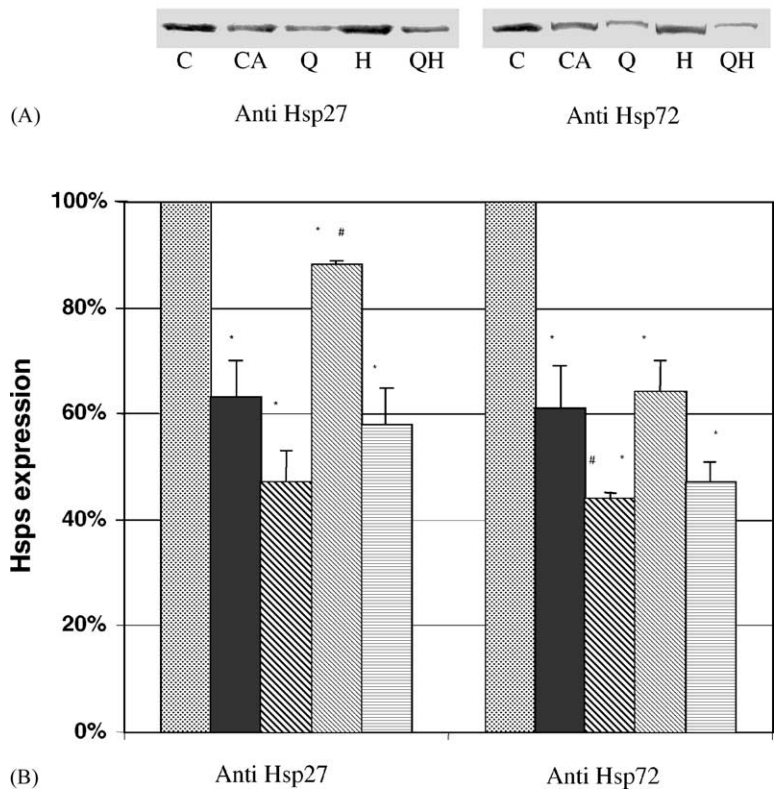

Fig. 1. The effect of quercetin and heat shock on Hsp27 and Hsp72 expression in HeLa cells transfected with anti-sense oligonucleotides anti-Hsp27 and anti-Hsp72 determined by Western blot analysis. (A) Representative Western blot of Hsp72 and Hsp27 expression. (B) Quantitive analysis of Hsp72 and Hsp27 expression. C: control of nontransfected cells; CA: control of transfected cells; Q: cells incubated only with quercetin for 7.5 hr; H: cells exposed to heat shock at 42° for 1 hr; and QH: cells pretreated with quercetin (15 μg/mL) for 4 hr at 37°, then exposed to hyperthermia and incubated for 2.5 hr at 37°. (∗) $P < 0.05$ in comparison to control of nontransfected cells. (#) $P < 0.05$ in comparison to control of transfected cells.

Table 3
Effect of quercetin and heat shock on apoptosis induction in HeLa cells transfected with anti-sense oligonucleotides blocking the expression of Hsp27 (anti-Hsp27) and Hsp72 (anti-Hsp72) estimated by comet assay method

Conditions	Apoptotic cells (%)	
	Anti-Hsp72	Anti-Hsp27
C	0.95 ± 0.07	0.95 ± 0.07
CA	2.80 ± 0.28*	1.10 ± 0.00
Q	51.85 ± 1.34*	44.30 ± 1.27*
H	4.2 ± 0.14*	8.75 ± 0.21*
QH	57.40 ± 1.55*	48.85 ± 0.49*

C: control of nontransfected cells; CA: control of transfected cells; Q: cells incubated only with quercetin for 7.5 hr; H: cells exposed to heat shock at 42° for 1 hr; and QH: cells pretreated with quercetin (15 μg/mL) for 4 hr at 37°, then exposed to hyperthermia and incubated for 2.5 hr at 37°.
* Values are significantly different from those of control at $P < 0.005$.

of Hsp27 and Hsp72 in comparison to nontransfected cells by about 39% in each case (Fig. 1). Additional treatment of studied cells with quercetin (15 μg/mL) resulted in increased inhibition of studied protein expression and was estimated at the average level of about 44%. Exposure of HeLa cells to heat shock itself resulted in weak induction of Hsp72 expression by about 3% and increased Hsp27 synthesis by about 25% in comparison to only transfected cells, but still it was lower than in nontransfected cells. Additional treatment with quercetin inhibited studied protein expression by about 30% for Hsp27 and 17% for Hsp72, in comparison to only heat shocked cells.

3.4. Apoptosis induction by quercetin and heat in transfected HeLa cells

The blockage of Hsp27 and Hsp72 gene expression with anti-sense oligonucleotides had weak impact on apoptosis induction in comparison to control cells. The comet assay method (Table 3) showed that significant increase in the amount of apoptotic dead cells was observed after quercetin treatment and accounted for about 52%, in relation to Hsp72 anti-sense transfection, and 44% after Hsp27 anti-sense transfection. In cells incubated with quercetin at first and then additionally exposed to 42° for 1 hr, the number of apoptotic cells was even greater. Heat shock alone increased the number of apoptotic cells by 4% after anti-Hsp72 oligonucleotide transfection and 9% in cells with blocked HSP27 gene, in comparison to the control.

4. Discussion

Normal development of cells and tissue homeostasis is regulated by proliferation, differentiation and death processes. Abnormal heat shock proteins expression has been identified as one of the deregulating factors. Many tumour cells, for example, exhibit constitutively elevated levels of Hsps, that protect them from harmful conditions and increase chemotherapy tumour resistance and poor prognosis for cancer patients [4,7,8,19,24].

Our earlier experiments [17,29] and other author's observations [4–6,11] indicated that quercetin acts as good inhibitor of heat shock proteins expression in several tumour cell lines. It was suggested that such effect has an impact on increased tumour cells sensitivity and in consequence, apoptosis induction. In this study, we present the results of experiments concerning apoptosis induction by quercetin in cervical cancer cells (HeLa) and involvement of heat shock proteins in this process.

Our experiments, based on two independent variants, using quercetin alone and in combination with 1 hr-long hyperthermia (42°), indicated increased apoptosis induction. Keeping in mind, that quercetin has a broad range of activities within cells, thus many ways of apoptosis induction by this flavonoid may exist. Treatment with flavonoid can cause a rapid induction of caspase-3 activity and stimulate proteolytic cleavage of poly-(ADP-ribose) polymerase (PARP). Furthermore, quercetin induces loss of mitochondrial transmembrane potential, induces elevation of reactive oxygen species (ROS) production and causes the release of mitochondrial cytochrome *c* into the cytosol, and subsequent induction of pro-caspase processing [12]. Alterations in mitochondrial membrane permeability and in consequence cytochrome *c* release is accompanied by accumulation of numerous free radicals, forming ROS and depletion of anti-oxidants, such as gluthatione (GSH). Generated oxidative stress stimulates the expression of Hsp70 and Hsp27, which limit cytochrome *c* release, apoptosome formation and procaspase-9 activation [7]. Inhibition of heat shock proteins may determine the alternative mechanism of the pro-apoptotic activity of quercetin. HeLa cells transfected with anti-sense oligonucleotides, anti-Hsp27 and anti-Hsp72 proved to be an ideal model to examine the contribution of heat shock proteins to the resistance to apoptosis. Our experiments demonstrate that transfected cells generally showed diminished heat shock proteins levels in comparison to control. Incubation of HeLa cells with quercetin for 7.5 hr resulted in additional inhibition of Hsp27 and Hsp72 expression. This can be explained by the effect of flavonoid on 40% of HeLa cells, in which the presence of anti-senses in nuclei were not observed. In the same way the induction of studied protein levels in heat shocked HeLa cells treated or not with quercetin in comparison to only transfected cells can be explained. The mechanism of inhibition of Hsp27 or Hsp72 expression by quercetin is not clearly understood and several possible explanations exist. One possible explanation suggests that quercetin inhibits Hsp expression at the level of transcription by preventing the Heat shock factor 1 and 2 (Hsf1 and Hsf2) binding to the conserved DNA sequence known as the Heat Shock Element (HSE) in the promoter region of *hsp* genes [11,15]. Other experiments have indicated that quercetin acts on early events before Hsp synthesis, by blocking the

additional modifications necessary for activation of Hsf, like posttranslational phosphorylation or by causing conformational changes of the factor, and inhibiting its interactions with other DNA-binding proteins in the promoter region [6,18]. Diminished Hsp27 expression by quercetin can be also explained by the ability of studied flavonoid to inhibit the activity of Hsp25 kinase, an enzyme necessary for the activation of small heat shock proteins [33].

Estimation of apoptotic cells by comet assay method in transfected HeLa cell line revealed that quercetin strongly increased the number of apoptotic cells in comparison to nontransfected cells. This effect was enhanced by additional exposure of studied cells to heat shock. Obtained results directly indicate that overexpression of Hsp27 and Hsp72 may be responsible for cell resistance to the induction of apoptosis. We can also conclude that reduced expression of Hsp27 and Hsp72 facilitates the synergistic effect of quercetin and hyperthermia on apoptosis. Hyperthermia by itself had little effect on apoptotic cell death in transfected HeLa cell line, therefore other factors than Hsps may be involved in the resistance of heat shocked cells to apoptosis induction.

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