Quantum dot imaging for HSP70 and HSF-1 kinetics in SCC-25 cells with or without leucine deprivation following heat shock

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Received December 14, 2012; Accepted March 4, 2013

DOI: 10.3892/or.2013.2372

Abstract. The aim of this study was to develop a quantum dot-based approach for heat shock protein 70 (HSP70) and heat shock factor 1 (HSF-1) kinetics following heat shock, and to discover approaches to thermotherapy based on disrupting the effect of activation of HSF-1 and the accumulation of HSP70 by leucine deprivation. SCC-25 cells cultured with limiting leucine or normal leucine were stressed at 42˚C for 30 min, and were cultured for 2, 4, 6, 8 and 10 h, respectively. The expression of HSP70 and HSF-1 was observed using confocal laser microscopy and semi-quantitative analysis was performed by Image-Pro Plus. At 6 h after heating, HSF-1 in cells cultured with normal leucine was activated and translocated from the cytosol to the nucleus, and the synthesis of HSP70 reached the maximum value and had a tendency to gather in the nucleus. However, in cells cultured with limiting leucine, HSF-1 activity decreased and accumulation of HSP70 was not found. Leucine deprivation results in the inactivation of HSF-1 leading to slight accumulation of HSP70 and no tendency to gather in the nucleus. Thus, HSF-1 may serve as a novel therapeutic target in the treatment of oral cancer.

Introduction

Oral squamous cell carcinoma (OSCC) is a common malignant tumor and the leading cause of oral cancer-related mortality (1-6). In China, the incidence of OSCC has been on a steady rise, and the thermotherapy targeted to heat shock protein 70 (HSP70) has a marked impact on OSCC treatment (7,8). HSP70 is known to assist the folding of nascent polypeptide chains, act as a molecular chaperone and mediate the repair and degradation of altered or denatured proteins. The ability of cells to respond to stress by increasing their HSP levels depends on the activity of heat shock factor (HSF). HSF-1 is known for its activation of transcription of the HSP genes during proteotoxic stress which can bind to the 50 promoter regions of all HSP genes and trigger instantaneous and massive transcription of these stress protein genes. The HSF-1/HSP system plays an important role in OSCC prognosis and treatment selection, being associated with a disparate range of tumor initiators and promoters. This system has thus emerged as a source for potential biomarkers of cell transformation and tumor progenesis. The status of HSP70 and HSF-1 is an integral part of the clinicopathological practice of OSCC, and accurate HSP70 and HSF-1 quantification and co-localization is crucial for treatment strategies (9).

The new semiconductor nanocrystals, quantum dots (QDs), are fluorescent semiconductor nanocrystals with a 2-10 nm core diameter, possessing several advantages over conventional fluorescent dyes, such as wide excitation spectra, significant photostability and a long fluorescence lifespan (10-18). These characteristics have attracted considerable interest in their application in immunohistochemistry for biomarker quantification and co-localization in oral tumors for prognosis and treatment (20-31). The present study sought to develop a QD-based approach for a long dynamic observation of physiological changes of HSP70 and HSF-1 in SCC-25 cells induced by heat shock, and to discover approaches to disrupt the influence of activation of HSF-1 and the accumulation of HSP70 in oral cancer.

Materials and methods

Materials. QDs (goat anti-mouse QD_{525nm}-IgG and goat anti-mouse QD_{655nm}-IgG) were purchased from Invitrogen. Mouse anti-human HSP70 antibody and mouse anti-human HSF-1 antibody was provided by Abcam. DMEM/F12 medium with or without leucine and trypsin were purchased from Sigma. The laser confocal microscope used was Leica TCS SP2, Germany.

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Key words: quantum dot, protein kinetics, heat stress, heat shock protein 70, heat shock factor 1, oral squamous cell carcinoma, leucine deprivation

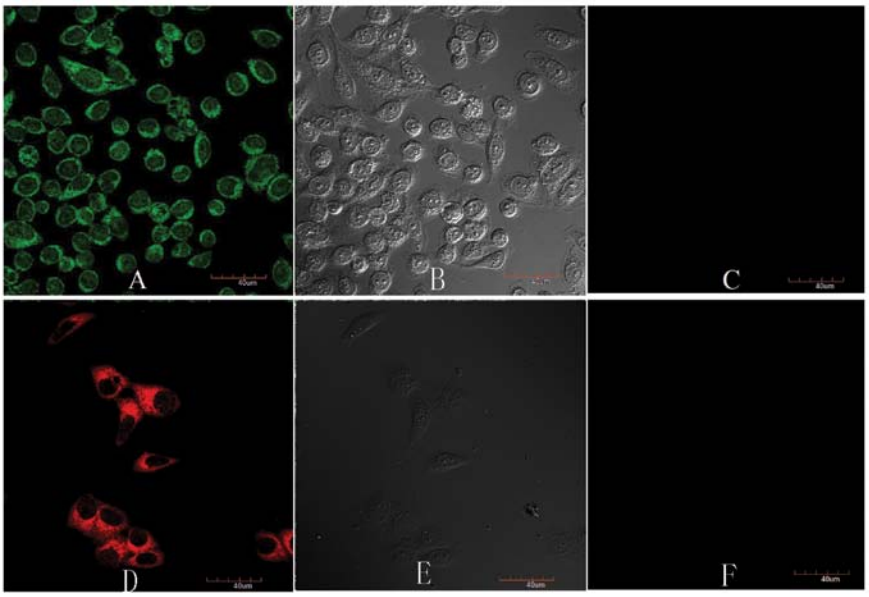

Figure 1. Fluorescence imaging of HSP70 and HSF-1 tagged by QD_{525nm} or QD_{655nm}. QD_{525nm}-marked HSP70 was clearly expressed in the cytoplasm and nucleus of SCC-25 cells, and was mainly distributed in the cytoplasm (A). The QD_{655nm}-marked HSF-1 was expressed in the cytoplasm (D).

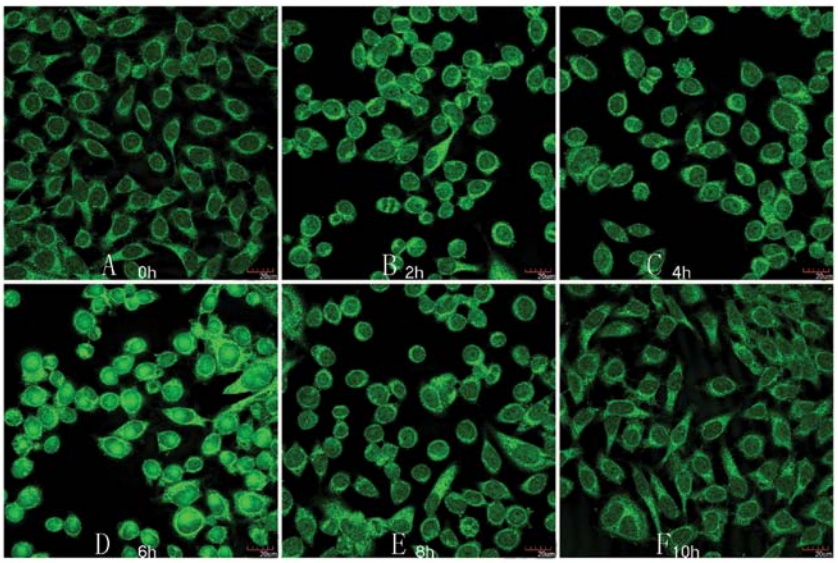

Figure 2. HSP70 kinetics in cells cultured with normal leucine following heat shock at (A) 0 h, (B) 2 h, (C) 4 h, (D) 6 h, (E) 8 h and (F) 10 h. The synthesis of HSP70 in cells cultured with normal leucine began to increase at 2 h (B), reached the maximum value and had a tendency to gather in the nucleus at 6 h (D).

Methods

Cell culture. For the cell culture, SCC-25 cells were cultured in DMEM/F12 medium (Sigma) with or without leucine and were placed in an incubator containing a 5% volume fraction of CO_2 at 37˚C. The culture medium was changed every 2 days, and 0.25% trypsin was used to digest and subculture the cells after 2-3 days.

Cell inoculation. For the cell inoculation, the digested cells were inoculated onto a confocal-dedicated utensil at a density of ~$1x10^5$ cells. After culturing for 1-2 days, the cells were 80% confluent.

Heat stress. For the heat stress (32), cells were stressed at 42˚C for 30 min (groups A, B, C, D, E and F). Following incubation at 37˚C constant temperature, immunofluorescence was performed on all groups after 0 h (group A), 2 h (group B), 4 h (group C), 6 h (group D), 8 h (group E) and 10 h (group F).

Cell fixation. For the cell fixation, all groups were washed twice with 0.01 mol/l (pH 7.4) TBS and were fixed with pre-chilled methanol for 10 min.

Immunofluorescence. For the immunofluorescence, the fixed SCC-25 cells were washed with TBS, incubated with Triton X-100 for 10 min and washed. They were then incubated at 4˚C overnight with mouse anti-human HSP70 antibody or mouse anti-human HSF-1 antibody (dilution ratios were 1:100). The cells were then washed 3 times in TBS and were incubated

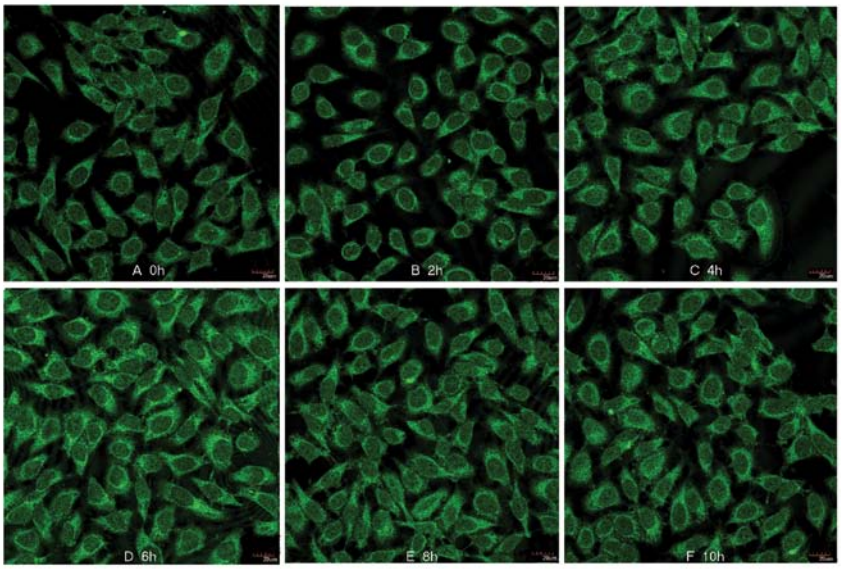

Figure 3. HSP70 kinetics in cells cultured with limiting leucine following heat shock at (A) 0 h, (B) 2 h, (C) 4 h, (D) 6 h, (E) 8 h and (F) 10 h. The synthesis of HSP70 in cells cultured with limiting leucine showed a litter increase at 6 h, but the accumulation of HSP70 had no tendency to gather in the nucleus (D).

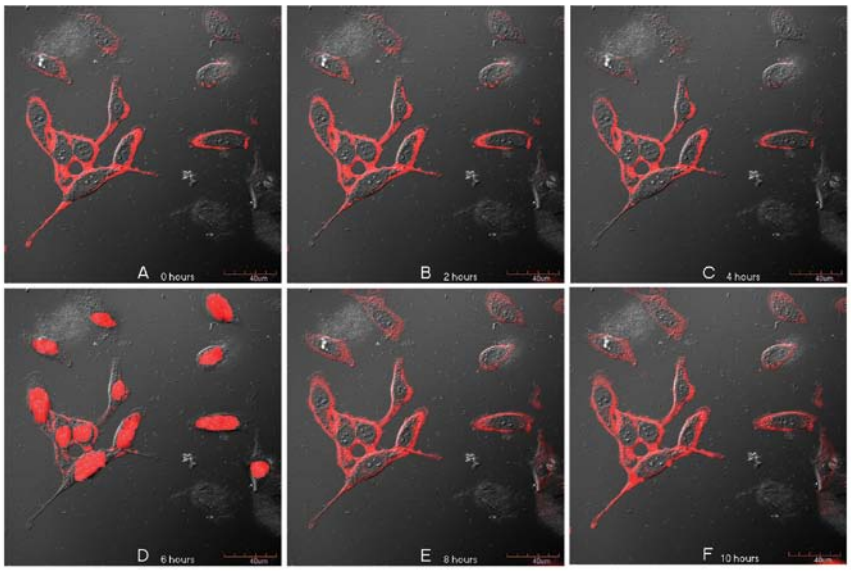

Figure 4. HSF-1 kinetics in cells cultured with normal leucine following heat shock at (A) 0 h, (B) 2 h, (C) 4 h, (D) 6 h, (E) 8 h and (F) 10 h. The majority of HSF-1 was localized in the plasma membrane cytoplasm at 0, 2, 4, 8 and 10 h. However, at 6 h after heating at 42˚C for 30 min, an increasing number of HSF-1 translocated from the cytosol to the nucleus, similar to the translocation of HSP70 (D).

with buffer solution in a 37˚C moist box for 10 min, followed by incubation with the equivalent mixed liquid of goat anti-mouse QD_{525nm}-IgG or goat anti-mouse QD_{655nm}-IgG (1:100) in a 37˚C moist box for 45 min. Finally, the cells were washed three times with TBST and sent for detection away from light. Each experiment was repeated 5 times.

Detection under laser confocal fluorescence microscopy and analysis by Image-Pro Plus. The wavelength of excitation spectra was 488 nm and the constant temperature system was adjusted to 30˚C.

Statistical analysis. Data are expressed as the means ± SD. Comparisons between two groups were conducted by one-way ANOVA. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

HSP70 and HSF-1 labeled by QD_{525nm} or QD_{655nm}. With a laser confocal microscopy, QD_{525nm}-marked HSP70 was clearly expressed in the cytoplasm and nucleus of SCC-25 cells, mainly distributed in the cytoplasm (Fig. 1A). The QD_{655nm}-marked HSF-1 was expressed in the cytoplasm (Fig. 1D).

HSP70 kinetics in cells cultured with normal leucine or limiting leucine. With a laser confocal microscopy, the synthesis of HSP70 in cells cultured with normal leucine

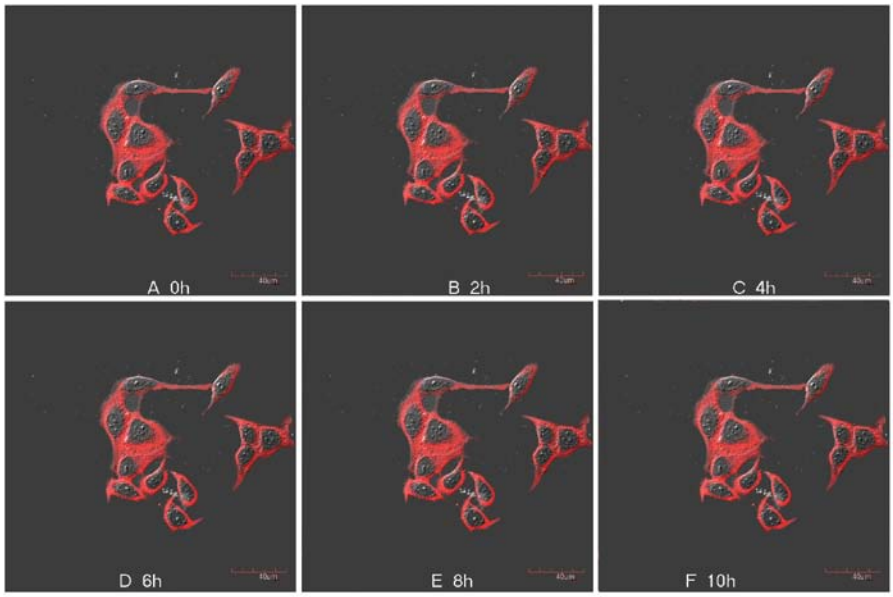

Figure 5. HSF-1 kinetics in cells cultured with limiting leucine during all the recovery periods of heat shock at (A) 0 h, (B) 2 h, (C) 4, h (D) 6 h, (E) 8 h and (F) 10 h. The lymphocyte positioning of HSF-1 in cells cultured with limiting leucine showed only slight change. HSF-1 translocation from the cytosol to the nucleus was not observed at 6 h after heat shock (D).

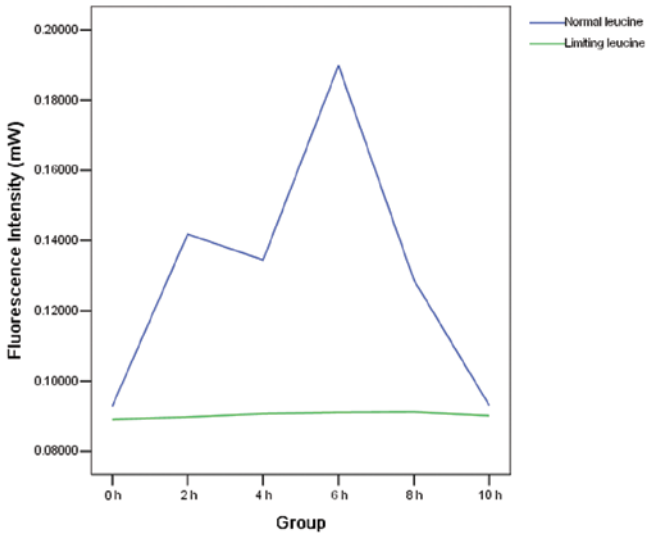

Figure 6. Semi-quantitative determination of HSP70 in cells cultured with limiting leucine or normal leucine during all the recovery periods of heat shock.

began to increase at 2 h (Fig. 2B), they reached the maximum value at 6 h, and had a tendency to gather in the nucleus at 6 h (Fig. 2D).

However, the synthesis of HSP70 in cells cultured with limiting leucine showed a small increase at 6 h (Fig. 3D). The accumulation of HSP70 showed no tendency to gather in the nucleus (Fig. 3D), whereas cells without leucine deprivation showed a translocation from the cytoplasm to the nucleus (Fig. 2D) at 6 h.

HSF-1 kinetics in cells cultured with normal leucine or limiting leucine. In an attempt to elucidate the difference of the HSP70 kinetics between the limiting leucine and the normal leucine group, we investigated the dynamic distribution of HSF-1 under laser confocal fluorescence microscopy.

The subcellular localization of HSF-1 in cells cultured with normal leucine was different during the recovery periods of heat shock (Fig. 4). The majority of HSF-1 was found to be localized in the cytoplasm at 0, 2, 4, 8 and 10 h after heating at 42˚C for 30 min (32). However, at 6 h, an increasing number of HSF-1 translocated from the cytoplasm to the nucleus (Fig. 4D), similar to the translocation of HSP70 (Fig. 2D).

By contrast, in cells cultured with limiting leucine, the subcellular localization of HSF-1 presented only a slight change, HSF-1 was localized in the cytoplasm during the recovery periods of heat shock, and no translocation was observed at 6 h after heat shock (Fig. 5D).

Semi-quantitative determination of HSP70 in cells cultured with limiting leucine or normal leucine during all the recovery periods of heat shock. HSP70 was detected in each group five times and 65 photofluorograms of each group were analyzed by Image-Pro Plus. Results are expressed as means ± SD.

As indicated in Table I and Fig. 6, the synthesis of HSP70 in cells cultured with normal leucine had a significant increase at 2 h after the heat shock ($P<0.05$). It reached the maximum value at 6 h (0.1898172±0.00219462 vs. 0.0928257±0.00193721 at 0 h; $P<0.05$), and it began to decrease at 8 h. At 10 h, it returned to the level at 0 h.

The synthesis of HSP70 in cells cultured with limiting leucine demonstrated a small increase during all the recovery periods of heat shock ($P>0.05$) (Table II and Fig. 6).

Concurrently, the synthesis of the HSP70 in cells cultured with normal leucine significantly increased compared to that of the HSP70 in cells cultured with limiting leucine at 2, 4, 6 and 8 h ($P<0.05$).

Table I. HSP70 expression kinetics in cells cultured with normal leucine during all the recovery periods of heat shock (0, 2, 4, 6, 8 and 10 h).

Group	0 h	2 h	4 h	6 h	8 h	10 h
Fluorescent intensity	0.0928257±0.00193721	0.1418270±0.00437687	0.1344760±0.00511160	0.1898172±0.00219462	0.1287666±0.00353873	0.0930118±0.00578231

Table II. HSP70 expression kinetics in cells cultured with limiting leucine during all the recovery periods of heat shock (0, 2, 4, 6, 8 and 10 h).

Group	0 h	2 h	4 h	6 h	8 h	10 h
Fluorescent intensity	0.0891216±0.00165493	0.0897608±0.00237068	0.0907190±0.00045941	0.0910551±0.00075798	0.0911681±0.01056427	0.0901642±0.00125764

Discussion

Heat shock proteins (HSPs) were first identified as stress proteins that confer resistance to physical stresses such as elevated temperatures in all cellular organisms (33-36). HSP70 expression becomes deregulated in oral cancer. Elevated HSP70 expression leads to resistance to subsequent thermotherapy.

In the present study, we developed the QD-IHC protocol for HSP70 kinetics in SCC-25 cells cultured with or without leucine following heat shock. At 6 h after heating at 42˚C for 30 min, the synthesis of the HSP70 in cells without leucine deprivation increased approximately 2-fold whereas that of cells with leucine deprivation showed only a small increase. Particularly the accumulation of HSP70 in cells cultured with normal leucine had a tendency to gather in the nucleus at 6 h whereas cells with leucine deprivation showed no translocation. Concurrently, the synthesis of HSP70 in the normal leucine group was increased significantly compared to that of the limiting leucine group at 2, 4, 6 and 8 h, respectively ($P<0.05$).

In an attempt to elucidate the mechanisms of HSP70 kinetics associated with leucine deprivation, we investigated the dynamic distribution of HSF-1 in SCC-25 cells cultured with or without leucine following heat shock. HSF-1 is known for its activation of transcription of the HSP genes during heat shock (37-42); it possesses a complex modular structure with several functional domains such as the DNA-binding domain (DBD; residues 15-120) and trimerization domains (residues 130-203). The trimerization domain is composed of three arrays of hydrophobic heptad repeats (HR-A/B). When the HSF-1 monomers come together, they form a leucine zipper, an artifact typically seen in dimerization. Once the heat shock response is induced, HSF-1 is activated through trimerization, accumulation in the nucleus, post-translational modifications and binding to HSP genes through DBD.

Leucine is one of the three branched chain amino acids (BCAA) along with isoleucine and valine. Leucine is the most prominent of the three and is the sole amino acid behind the branched chain amino acid stimulation of protein synthesis and anti-catabolic actions. Leucine deprivation results in the inactivation of HSF-1, and leads to a sharp decrease in the transcript level of HSF-1 target genes such as HSPA1A (HSP70), DNAJB1 (HSP40) and HSP90AA1 (43).

In leucine-deprived cells, HSF-1 loses its DBD activity and the HSF-1 monomer trimerization, accumulation in the nucleus and post-translational modifications were all inhibited. Leucine deprivation results in the inactivation of HSF-1, leading to slight accumulation of HSP70 in the nucleus.

With the valuable research tools for tumor prognosis and treatment (44-47), protein kinetics with QD provide a rationale for thermotherapy accompanied by short-term dietary restriction of leucine in patients with oral tumor.

In conclusion, the HSF-1/HSP system is implicated in several crucial steps in oncogenesis and tumor progression, activated HSF-1 and increased HSP levels may aid in the oncodiagnosis and treatment in the clinic. Leucine deprivation can disrupt the influence of activation of HSF-1 and the accumulation of HSP70 following heat shock. Thermotherapy accompanied by short-term dietary restriction of leucine may be a novel approach for the treatment of oral cancer.

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