The role of heat shock protein 70 in the thermoresistance of prostate cancer cell line spheroids

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Received 5 January 2004; accepted 27 January 2004

First published online 23 February 2004

Abstract

Heat shock protein 70 (Hsp70), a protein induced in cells exposed to sublethal heat shock, is present in all living cells and has been highly conserved during evolution. The aim of the current study was to determine the role of heat shock proteins in the resistance of prostate carcinoma cell line spheroids to hyperthermia. In vitro, the expression of Hsp70 by the DU 145 cell line, when cultured as monolayer or multicellular spheroids, was studied using Western blotting and enzyme-linked immunosorbent assay methods. The level of Hsp70 in spheroid cultures for up to 26 days at 37°C remained similar to monolayer cultures. However, in samples treated with hyperthermia at 43°C for 120 min, the spheroid cultures expressed a higher level of Hsp70 as compared to monolayer culture. Under similar conditions of heat treatment, the spheroids showed more heat resistance than monolayer cultures as judged by the number of colonies that they formed in suspension cultures. The results suggest that cells cultured in multicellular spheroids showed more heat resistance as compared to monolayer cultures by producing higher levels of Hsp70.

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Key words: Hyperthermia; Multicellular spheroid; Heat resistance; DU 145 prostate cell line; Heat shock protein 70

1. Introduction

Hyperthermia is considered to be a potent procedure for cancer treatment [1,2]. The heat sensitivities of cancer cells vary widely, probably due to differences in both intrinsic heat sensitivity and tolerance development [3]. In vitro experiments, in vivo animal studies, and clinical trials have revealed that hyperthermia may serve as a powerful tool in the treatment of prostate cancer [4-6].

At the cellular level, hyperthermic stress induces the synthesis of a class of proteins termed ‘heat shock proteins’ (Hsp) [7]. The Hsps are acidic proteins which range in molecular weight from 8 to 110 kDa [8]. Hsps have been reported to be cytoprotective and this function has been attributed to Hsp70 [9,10], a member of the 70 kDa family of Hsps. Hsp70 has been shown to confer resistance to radiation and hyperthermic treatment for recurrent breast cancer [11]. It has been shown that overexpression of Hsp70 generally confers heat resistance to cancer cells, suggesting that Hsp70 has a protective function against thermal stress, in addition to its functions in the repair and recovery of heat-damaged cells [9,12].

The use of spherical cell aggregates or spheroids in biological research began during the first half of the past century [13]. Multicellular tumor spheroids are tumor cell aggregates grown from one or several cell clones. This in vitro model has a three-dimensional structure that mimics micro-tumors or metastases and some of their properties [14]. Multicellular tumor spheroids have been used as experimental models in the study of tumor cell metastasis [15]. The spheroid model system offers many of the advantages in terms of experimental manipulation and analysis that are inherent in monolayer tissue cultures, yet it exhibits many of the properties seen in prevascularized tumors growing in vivo [16].

It has been suggested that there may exist some physiological differences between cells grown in monolayer and cells grown in multicellular tumor spheroids [17-19]. It has been shown that spheroid cultures of human prostate cancer cells may provide unique insights regarding cell adhesion and apoptotic potential that are diminished or absent in monolayer cultures [20]. It has been shown that growth of human glioma cells in these two systems led to a different degree of sensitivity to radioiodinated iododeoxyuridine [21]. Several authors have reported higher radio- and thermoresistance of cells in spheroids compared with monolayer cultures [22,23].

In this study we have determined the role of Hsp70 in the resistance of prostate carcinoma cell line [24] spheroids to hyperthermia. DU 145 is an established cell line that can self-assemble into large, stable spheroids through a combination of intracellular communication and diffusion [25]. We have studied the influence of three-dimensional contact between DU 145 cells on their sensitivity to hyperthermia at 43°C as compared with monolayer cultures. Then we measured the level of Hsp70 expression in DU 145 spheroids compared to monolayer level and studied the effect of hyperthermia at 43°C on Hsp in these two models of culture.

2. Materials and methods

2.1. Cell line

Human prostate carcinoma cell line DU 145 was maintained in RPMI 1640 (Gibco) supplemented with 10% fetal calf serum (FCS) (obtained locally), 500 U/ml of penicillin (Sigma) and 200 mg/l of streptomycin (Jaberebn-Hayan).

Abbreviations: Hsp70, heat shock protein 70; FCS, fetal calf serum; PBS, phosphate-buffered saline; CFA, colony-forming ability

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2.2. Monolayer culture

Cells were cultured as monolayer at a density of $10^4$ cells/cm$^2$ in RPMI 1640 tissue culture flasks (Nunc). Cultures were maintained at 37°C in a humidified atmosphere of 7.5% CO$_2$. Cultures were propagated or cells were harvested by trypsinizing cultures with 1 mM EDTA/0.25% trypsin (w/v) in phosphate-buffered saline (PBS).

2.3. Spheroid culture

Spheroids were initiated using the liquid overlay technique [26]. $5 \times 10^5$ cells were seeded into 100 mm Petri dishes coated with a thin layer of 1% agar (Bacto Agar, Difco, Detroit, MI, USA) with 10 ml of RPMI 1640 supplemented with 10% FCS. The plates were incubated at 37°C in a humidified atmosphere of 7.5% CO$_2$. Half of the culture medium was replaced with fresh medium twice per week.

2.4. Heat treatment of monolayer cultures

Cells were cultured at $5 \times 10^5$ cells per flask in T-25 culture flasks in RPMI 1640 culture medium supplemented with 10% FCS for monolayer culture. After 48 h, the culture medium was replaced with RPMI 1640 culture medium which was used during the period of heat treatment. Hyperthermia was applied at 43°C for 120 min in a precision water bath (Hakke F3) with ±0.1°C accuracy. Controls were exposed to 37°C. The cells were then harvested, counted, and tested for viability as described above. The cells were assayed for colony-forming ability (CFA) by plating 500 cells per dish in 60 mm Petri dishes. The colonies were counted on day 10. The cells were also used for measuring the level of Hsp70 after various times of incubation.

2.5. Heat treatment of spheroid cultures

To study the effect of hyperthermia on spheroids, cells were cultured at $5 \times 10^5$ cells per 100 mm dishes coated with a thin layer of 1% agar for multicellular spheroid formation. On day 11, the spheroids were gently precipitated. The culture medium was replaced with RPMI 1640 medium and spheroids were resuspended and heat-treated as described for monolayer cultures. They were then treated with 300 μl of PBS containing 1 mM EDTA/0.25% trypsin (w/v) for 10 min at 37°C. Trypsin was neutralized by addition of 700 μl of culture medium containing FCS. Half of the spheroids were mechanically disaggregated. Single cells were counted and tested for viability. Cells were then seeded at a density of 3000 cells per Petri dish for colony formation assay. The other half was used to measure the level of Hsp70, after various times of incubation.

2.6. Clonogenic assay

Single cell suspensions, from either spheroid or monolayer cultures, containing 3000 and 500 cells respectively, were seeded in 60 mm Petri dishes (Nunc) and grown in RPMI 1640 supplemented with 10% FCS. The cells were incubated at 37°C in a humidified atmosphere of 7.5% CO$_2$ for 10 days. The colonies were counted using an inverted phase microscope (Zeiss, Axiovert 405M) on day 10 and the plating efficiency was determined.

2.7. Survival curves

Survival curves were generated by plotting the log of the ratio of the number of colonies formed at a given heating condition to the number of colonies produced by related control cells versus the heating time at the given temperature.

2.8. Protein isolation and analysis

Cells were washed with PBS and lysed at 4°C for 30 min in 100-200 μl of lysis buffer (10 mM Tris-HCl pH 6.8, 100 μg/ml phenylmethylsulfonyl fluoride, 0.14 M NaCl, 1.5 mM MgCl$_2$ and 0.5% NP40) per 100 mm dish. The lysate was then centrifuged at 12000 rpm for 20 min to pellet large cellular debris. Protein concentrations were measured using the Bradford method [27]. Equal amounts of samples (50 μl) were mixed with an equal volume of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, heated at 90°C for 5 min then loaded onto 10% SDS-PAGE for electrophoresis.

2.9. Western blotting

To perform the Western blot analysis, the separated proteins were electroblotted to nitrocellulose (Schleicher and Schuell) and then probed with polyclonal anti-Hsp70 (Dako). The primary antibody was detected with secondary anti-rabbit IgG conjugated with horse-radish peroxidase to generate a 4-chloro-1-naphthol signal (Sigma). To help in identification, protein standard of recombinant rat Hsp70 protein (Stressgen) was included on the 10% SDS-PAGE. The protein standard was visible on the nitrocellulose Western blot. The antibody-labeled Hsp70 polypeptide band on the developed blots was digitally captured with a UVI Tec and band intensities were measured using Totallab$^\text{TM}$ software.

2.10. Enzyme-linked immunosorbent assay (ELISA)

After hyperthermia, cells were incubated for various times and then harvested as described above. Cells were washed with PBS and lysed with 1 ml of lysis buffer for 30 min at 4°C. Cell lysates were centrifuged at 12000 rpm for 20 min and the supernatant was saved for the sandwich enzyme immunoassay using a Hsp70 ELISA kit (Stressgen).

2.11. Statistical analysis

Data are given as mean values ± S.E.M., with ‘n’ denoting the number of experiments. Student’s t-test was applied as appropriate. A value of $P \leq 0.05$ was considered significant.

3. Results

3.1. Cell characteristics

The DU 145 prostate carcinoma cell line grows as a monolayer on plastic culture flasks with a population doubling time of approximately 23 h. These cells could survive in low population densities and form colonies with at least 50 cells over 10 days. The DU 145 cells could form spheroids in liquid overlay cultures.

3.2. Basal levels of Hsp70 protein during spheroid development

We compared the Hsp70 levels in logarithmic and plateau phases of growth of monolayer and spheroid cultures on various days of culture between day 1 and day 20 by SDS-PAGE and Western blotting as described above. From day 1 to day 20 of culture, the levels of Hsp70 from spheroid cultures re-

![A: Western blot showing basal levels of Hsp70 protein during spheroid development. B: Results of scanning of bands by Totalab$^\text{TM}$ software. Lanes are as follows: lane 1 = monolayer in logarithmic phase, 2 = monolayer in plateau phase, 3 = day 1, 4 = day 3, 5 = day 7, 6 = day 11, 7 = day 15, 8 = day 20 of spheroid culture, 9 = Hsp70 protein standard. Mean ± S.E.M. of three experiments.](image-url)
mained consistent as compared to monolayer samples with no apparent decrease or increase observed (Fig. 1). Therefore, we used the day 11 spheroid cultures, with a mean diameter of 100 μm, for further experiments.

3.3. Effect of hyperthermia on CFA

The response of spheroids to hyperthermia was studied by applying hyperthermia at 43°C for various periods of time to 11 day spheroids and monolayer cultures. The number of colonies formed by cells from monolayer and 11 day spheroids as a function of heating time is shown in Fig. 2A. As can be seen, hyperthermia reduced the CFA of both monolayer and spheroid cultures. However, the extent of reduction in the number of colony-forming cells from spheroids was much less than monolayer cultures.

The plot of survival fraction versus heating time at 43°C for monolayer and 11 day spheroid cultures is shown in Fig. 2B. This figure clearly shows that cells from spheroid cultures are more heat-resistant than cells from monolayer cultures and the extent of thermal resistance is dependent on the time of heating.

3.4. Effect of hyperthermia on spheroid and monolayer Hsp70 levels

3.4.1. Western analysis. The relationship between spheroid thermal resistance and the level of Hsp70 induction was studied by applying hyperthermia at 43°C for 120 min to monolayer and 11 day spheroid cultures. Levels of Hsp70 were measured over the first 7 h after exposure to hyperthermia. The results of Western blotting showed that 7 h after heat treatment the expression of Hsp70 was increased. The increase was statistically significant (Fig. 3).

3.4.2. ELISA analysis. The comparison of Hsp70 expression following hyperthermia in these two models of culture was further studied by measuring the Hsp70 in the samples as described above with an ELISA Hsp70 kit. As can be seen in Fig. 4, the expression of Hsp70 was significantly increased following hyperthermia 7 h after treatment in spheroid cultures as compared to monolayer cultures.

4. Discussion

Because of the particular architectural characteristics of multicellular tumor spheroids, it is demonstrated that this model of culture can be extremely useful in radiotherapy
The aim of the current study was to investigate various biochemical mechanisms involved in prostate malignancies. Prostate cells spheroids have been used extensively as a model system to study tumoral communication. The results are expressed as the ratio of HSP70 protein/total protein. The main interest of this model is the structural similarity with the tumor, which implies the presence of various gradients (such as oxygen, nutrients or pH) and intercellular communication.

Many cell lines can form multicellular spheroids. This property is highly dependent on the adhesion molecules such as the integrin and adherin families. Prostate cell spheroids have been used extensively as a model system to study various biochemical mechanisms involved in prostate malignancies. The aim of the current study was to investigate the thermal properties of human prostate cell line spheroids as compared to monolayer cultures with regard to the level of Hsp70 production by these two culture systems. The results of various days of spheroid culture showed that in the DU 145 cell line spheroids produced nearly the same amount of Hsp70 during the 26 days of study. Cells in the spheroid cultures showed resistance to hyperthermia at all levels of heat exposure. Under the same experimental conditions, spheroids produced higher levels of Hsp70 as compared to monolayer cultures. Therefore, the acquired thermal resistance of spheroid cultures may be attributed to the higher level of Hsp70 production.

In conclusion, this study showed that spheroid formation is not a stress-generating process as compared to monolayer culture of cells. Cells in the spheroid culture produced nearly the same amount of Hsp70 during the 26 days of study. Cells in the spheroid cultures showed resistance to hyperthermia at all levels of heat exposure. Under the same experimental conditions, spheroids produced higher levels of Hsp70 as compared to monolayer cultures. Therefore, the acquired thermal resistance of spheroid cultures may be attributed to the higher level of Hsp70 production.

Acknowledgements: This work was supported by grants from the Research Council of the University of Tehran. We wish to acknowledge the valuable advice and critical discussions of Dr. A.R. Shirazi.

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