Effects of thrombospondin antibody on the recovery of endothelial cells from hyperthermia

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Summary

In addition to the increased synthesis of the classical heat-shock proteins (28 000, 71 000, 73 000, 90 000 and 100 000 M<sub>r</sub> polypeptides) there is also an increase of thrombospondin in the growth medium of endothelial cells exposed to hyperthermia. The effect of a monoclonal antibody to thrombospondin on the recovery of endothelial cells from hyperthermia as it relates to cytoskeletal organization and cell spreading was assessed. The antibody interacts with the heparin-binding domain of thrombospondin in the extracellular matrix of cells. We report that during recovery from thermal insult at 37°C, intermediate filaments, stress fibres and microtubules show distinct time-recovery characteristics in bovine aortic endothelial cells; that in the presence of this antibody the cytoskeleton is notably altered; that this antibody causes retraction of endothelial cell processes; and that the recovery of the cytoskeleton in endothelial cells exposed to hyperthermia is prevented by the thrombospondin antibody in the time frame examined. Our data suggest that the recovery of cells from heat shock requires the integrity of thrombospondin and its interactions.

Key words: heat shock, endothelial cells, cytoskeleton, thrombospondin.

Introduction

Cells of most eukaryotes respond to mild heat stress by the induction of a set of polypeptides referred to as 'heat-shock proteins' (HSP). These polypeptides can also be induced by a variety of other stresses, including glucose deprivation (Ashburner and Bonner, 1979) and anoxia (Schlesinger et al. 1982). The response was first reported in Drosophila and the induction process has been studied extensively in these cells (Ashburner, 1982; for review). In Drosophila, the HSP have distinct induction characteristics and their expression is regulated at both transcriptional and translational levels to produce HSP as rapidly as possible.

Most heat-shock proteins are present under control conditions and appear to play essential functions in stressed and unstressed cells (Zimmerman et al. 1983; Bensaude et al. 1983; Morimoto and Fodor, 1984). Briefly, the abundant constitutive form of HSP70 family, HSP73, has been shown to have ATP-dependent uncoating activity on clathrin-coated vesicles in both Drosophila and mammals (Craig, 1985; Ungewichel, 1985). Recently, it has been shown that one important function of the HSP70 family appears to be ATP-dependent protein folding (Dehais et al. 1988; Chiro et al. 1988). The HSP90 transiently associates with tyrosine kinases and steroid hormone receptor complex. The HSP 100 synthesis increases during glucose/calcium deprivation (Welch et al. 1983). The HSP80 shows homology to immunoglobulin-heavy chain binding protein (Munro and Pelham, 1986; Haas and Wabl, 1983) and its synthesis also increases during glucose/calcium deprivation. Increased phosphorylation of HSP28 has been observed in response to mitogens and tumor promoters (Welch, 1985).

Previously, we have shown (Ketis et al. 1988) that in addition to increased synthesis of the classical HSP there is an increase of the thrombospondin (TS) polypeptide in the growth media and an increase in the message levels for TS in cells exposed to hyperthermic challenge followed by a recovery at 37°C. Recently, it has been reported by Donoviel et al. (1988) and Laherty et al. (1989) that the TS promoter shares two motifs with the promoter of HSP70, that is, it has a purine-rich sequence resembling closely the sequence required for serum stimulation of HSP70 and a sequence that resembles the heat shock response element (consensus sequence) of human and Drosophila HSP70. Thus our example is the first in the literature of an extracellular matrix protein that responds to heat shock (Ketis et al. 1988).

Thrombospondin is a trimeric glycoprotein of apparently identical subunits that is secreted from α-granules after platelet activation and may mediate platelet aggregation (George et al. 1980; Phillips et al. 1980). Platelet thrombospondin has been reported to support adhesion of erythrocytes parasitized with malaria and may mediate...
adherence of parasitized erythrocytes to melanoma and endothelial cells (Roberts et al. 1985, 1987). Thrombospondin is synthesized and secreted by a variety of cells in culture and is incorporated into the extracellular matrix of some cell lines (e.g. see Asch et al. 1986; Jaffe et al. 1985, 1983; McPherson et al. 1981; Raugi et al. 1982). Thrombospondin also supports the attachment of human squamous carcinoma cells, human melanoma cells, human platelets, human fibroblasts, human and bovine endothelial cells, human arterial smooth muscle cells, porcine epithelial cells and human U937 cells (McPherson et al. 1981; Roberts et al. 1987; Phillips et al. 1980; Jaffe et al. 1983; Raugi et al. 1982; Lawler et al. 1988). There appears to be considerable variation in cell spreading on thrombospondin for various cell lines (Roberts et al. 1987; Varani et al. 1986; Lawler et al. 1988). Roberts et al. (1987) have shown that the amino-terminal heparin-binding domain of thrombospondin is required to support spreading of human C361 melanoma cells on thrombospondin. On the other hand, the 18,000 Da carboxy-terminal domain is required for cell attachment. Thrombospondin has been shown to have a binding domain for heparin (Dixit et al. 1983; Lawler et al. 1985; Lawler and Slattery, 1981), fibronectin (La Vaugh et al. 1982), fibrogen (Leung and Nachman, 1982), collagen (La Vaugh et al. 1982; Mumbledy et al. 1984), plasminogen (Silverstein et al. 1984) and plasminogen activator (Silverstein et al. 1985). Recently, Lahav et al. (1982, 1984) have provided evidence for thrombospondin-fibronectin interaction during platele adhesion.

In an effort to understand the role of thrombospondin in the growth and proliferation of endothelial cells as it may relate to (patho-) physiological stress, and how this stress may affect the surrounding vascular milieu (specifically, smooth muscle cells) we examined the effect of a monoclonal antibody raised against human platelet TS in the recovery of the cytoskeleton in endothelial cells from hyperthermic stress. Our data suggest that the recovery of cells from hyperthermia requires the integrity of TS and its interactions. We speculate that TS may be involved in the recovery of endothelial cells from a stressed or injured state.

Materials and methods

Endothelial cells and cultures

Bovine aortic endothelial cells (BAEC) were isolated according to methods described by Booyse et al. (1975) and cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum and 2.5% Nu-serum (Collaborative Research, Wal-tham, MA) in the presence of antibiotics: penicillin, 100 µg ml⁻¹; streptomycin, 100 µg ml⁻¹; and amphotericin, 0.25 µg ml⁻¹. Parent cells were cultured and subsequently cloned in the same growth media. Cloned bovine aortic endothelial cells (BAEC) used in this study were passage five. Cells were identified as endothelium morphologically by their cobblestone appearance (Booyse et al. 1975), immunologically by staining with fluorescein labelled antifactor VIII (Booyse et al. 1975), and enzymatically by assay of angiotension II-converting enzyme.

Endothelial cells were grown on coverslips to 4 x 10⁴ to 6 x 10⁴ cells per coverslip. BAEC were exposed to desired experimental conditions and washed twice with Hank's balanced salt solution buffered with Hepes (15 mM), pH 7.4. The cells were then exposed to either thrombospondin or fibronectin antibodies at about 10 µg ml⁻¹ in DMEM without serum for up to 120 min at 37°C.

Immunofluorescence microscopy

Immunofluorescence staining was performed as described by Lazearides and Weber (1974). Fixed and permeabilized cells were incubated with primary antibody followed by the second antibody, rhodamine-labelled goat anti-mouse IgG (Cooiper Biomedical; Malvern, PA). A mouse monoclonal antibody against human fibronectin (IgG₁), was purchased from Calbiochem-Behring, Corporation (San Diego, CA); mouse monoclonal anti-tubulin antibody was from Amersham International plc. (Amersham, UK); mouse monoclonal anti-vimentin antibody was from ICN Immuno Biologicals (Lisle, IL); rhodamine-phalloidin was from Molecular Probes Inc. (Eugene, OR); and mouse monoclonal antithrombospondin immunoglobulin (IgG₂), was as described previously (Ketis et al. 1988b).

The monoclonal antibody (IgG₁) to fibronectin had no effect on cell spreading. The fibronectin antibody was the same IgG class as the thrombospondin antibody.

Cell integrity

Cell integrity was assessed by Trypan Blue exclusion and ⁴¹Cr release from endothelial monolayers as used previously (Ketis et al. 1988b).

Results

Altersations in the cytoskeletal elements after exposure to hyperthermia

In order to study the effect of TS antibody on the recovery of endothelial cells from hyperthermia as it relates to cytoskeletal organization and cell spreading, it was necessary to first examine the effects of heat treatment on the cytoskeleton of BAEC. The cytoskeletal collapse is well documented in several cell types (Glass et al. 1985; Cross et al. 1982; Thomas et al. 1982) other than endothelial, and the temporal analysis in BAEC was required to control for the effect of TS antibody. Thus, immunofluorescence studies were carried out on BAEC during normal growth conditions (Fig. 1A, C and E) and after heat treatment (Fig. 1B, D and F). When cells were exposed to 43°C for 3 h an alteration in the cytoskeletal organization was observed. Fixed and permeabilized BAEC from normal growth conditions stained for intermediate filaments (vimentin) (Fig. 1A), showed a network of wispy, wellspaced filamentous structures. Upon exposure to heat treatment, there was a collapse of intermediate filaments about the nucleus (Fig. 1B). Concomitantly, the stress fibers were destroyed (Fig. 1C versus D). There appeared to be a decrease in the number of actin filaments with an increase in intensity of staining at the periphery of the cell and some cells showed aggregated fluorescent material within the cell (Fig. 1C versus D). In addition, there was partial reorganization of the tubulin network (Fig. 1E versus F). The microtubules appeared to be clustered more about the nucleus (Fig. 1F). No loss of cell viability was apparent under this hyperthermic treatment.

Altersations in the cytoskeletal elements during recovery from hyperthermia

To analyze the distribution of the cytoskeletal elements upon recovery from hyperthermic treatment, BAEC were subjected to heat stress at 43°C for 3 h, followed by recovery at 37°C for up to 120 min (Fig. 2). Recovery or reorganization of the vimentin cytoskeleton to its native appearance (Fig. 1A) was complete when cells were allowed to recover at 37°C for 120 min (Fig. 2G). Actin filament reassembly appeared complete within 30 min at 37°C (Fig. 2E) and the microtubule organization appeared completely restored within 10–30 min at 37°C (Fig. 2F, J).
Effects of TS antibody on BAEC at 37°C

We used a well-characterized monoclonal antibody to TS (Lawler et al. 1985) to assess its effect on cytoskeletal organization and cell spreading of BAEC under control conditions. Continued exposure of cells to antibody for 30 min (Fig. 3D) resulted in partial collapse of the vimentin cytoskeleton and noticeable retraction of cell processes. When these cells were exposed to antibody for up to 120 min (Fig. 3G), spreading was dramatically inhibited and the vimentin cytoskeleton collapsed about the nucleus. Concomitantly, the number of stress fibers decreased with increase in time of exposure and there were punctate deposits of fluorescence within the cells. The most dramatic response to exposure of TS antibody at 37°C was noted in cells that were stained for microtubules. Within a 10 min exposure to antibody, the microtubule network appeared to retract from the cell membrane (Fig. 3C, arrows) and there appeared to be a notable decrease in the number of microtubules in the cytosol. Within a 30 min exposure to antibody (Fig. 3F) the fluorescence appeared more clustered about the nucleus, and by a 120 min exposure (Fig. 3I) the microtubules had disappeared altogether and there was increased appearance of aggregated material in the cytosol. The BAEC exposed to TS antibody for up to 120 min were determined to be viable by Trypan Blue exclusion and 51 Cr-release experiments.

Effects of TS antibody on BAEC recovery from hyperthermia

In order to study the effects of TS antibody on the recovery of the cytoskeleton in endothelial cells from hyperthermia, BAEC were exposed to test treatment and returned to 37°C for up to 120 min in the presence of TS antibody. During the first 10 min of the recovery period in the presence of antibody (Fig. 4A, B and C), the cytoskeleton (vimentin, actin, tubulin) responded similarly to that of heat-shocked cells allowed to recover for the same period in the absence of antibody (Fig. 2A, B and C). However, in heat-shocked cells allowed to recover for 30 or 120 min at 37°C in the presence of antibody, the cytoskeletal elements (vimentin, actin, tubulin) (Fig. 4D-I) appeared to be better dispersed throughout the cytosol and the reversal of spreading appeared less prominent as compared to cells at 37°C exposed to TS antibody (Fig. 3D–I). The heat-shocked cells that were allowed to recover at 37°C for up to 120 min in the presence of antibody appeared to present more actin (Fig. 4H) and microtubule (Fig. 4I) structures than cells at control temperatures exposed to antibody for hyperthermia.

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Fig. 2. Changes in cytoskeletal structure during recovery of BAEC from hyperthermia. BAEC were grown on 16-mm glass coverslips. Fixed and permeabilised cells were stained for vimentin (A,D,G), actin (B,E,H) and tubulin (C,F,I) cytoskeletal proteins using antibodies specific to each protein. Immunofluorescence micrographs, BAEC exposed to HS and allowed to recover at 37 °C for 10 (A,B,C) 30 (D,E,F) and 120 min (G,H,I).

the same test period (Fig. 3H and I). In addition, heat-shocked cells that were allowed to recover at 37 °C in the presence of TS antibody for up to 120 min (Fig. 4) were unable to reassemble totally their cytoskeleton, whereas heat-shocked cells allowed to recover for the same time interval but in the absence of TS antibody did reassemble their cytoskeleton (Fig. 2).

Discussion

The data presented show that a specific antibody raised against human platelet thrombospondin disrupts the vimentin, actin and tubulin cytoskeletal networks in endothelial cells at 37 °C and that the recovery of the cytoskeleton in endothelial cells from heat shock is prevented by the presence of this thrombospondin antibody. The thrombospondin antibody used in the study binds to the thrombospondin heparin-binding domain (Lawler and Hynes, 1986) and causes the retraction of endothelial cell processes (Figs 3, 4). It has been reported by Roberts et al. (1987) that the heparin-binding domain must be present to support cell spreading in human melanoma cells. Recently, one of us (J.L.) has shown that the amino acid sequence of thrombospondin has an Arg-Gly-Asp-Ala (RGDA) sequence (Lawler and Hynes, 1986) and that the GPIIb–IIIa-like complex on the surface of endothelial and smooth muscle cells functions as an RGD-dependent receptor for thrombospondin (Lawler et al. 1988). These data, with those of Cheresh (1987) and Cheresh and Spiro (1987), suggest that the thrombospondin-like vitronectin, fibrinogen and von Willebrand factor interact with a common integrin receptor on endothelial cells. The globular regions at both the amino-terminal and carboxy-terminal of thrombospondin have been reported to be involved in platelet aggregation or cell attachment and spreading (Lawler and Hynes, 1986; Roberts et al. 1987). As stated above the monoclonal antibody raised against human platelet thrombospondin used in this study, interacts with the heparin-binding domain of thrombospondin in the extracellular matrix of endothelial cells. This interaction may be translated into a conformational
change for thrombospondin or may result in clustering of receptors. The change then most likely provides a signal through transmembrane integrin receptors (Lewler et al. 1988) to the cytoskeleton. Integrin receptors have been shown in other cells to connect with cytoskeletal elements (Fox et al. 1984; Loftus and Albrecht, 1984). In the chick integrin complex (Chen et al. 1985; Damsky et al. 1985) the β1-subunit, co-distributes with actin, vinculin, talin, α-actinin and tropomyosin. Thus it appears that integrin receptors may serve as transmembrane 'integrators' of information from extracellular components to the cytosol and cytoskeleton. This then could be reflected by the collapse in the actin, tubulin and vimentin cytoskeletal elements that is observed in bovine aortic endothelial cells exposed to thrombospondin antibody for up to 120 min (Figs 3, 4). At this time, there is insufficient information in the literature (Chen et al. 1985; Damsky et al. 1985) for us to decide what cytoskeletal polypeptides are associated with integrin receptors, other than that described herein.

It is interesting to note that bovine aortic endothelial cells exposed to heat stress followed by a recovery at 37°C in the presence of thrombospondin antibody (Fig. 4) show overall less retraction of cell processes and maintain greater integrity of the vimentin, actin and tubulin cytoskeletal networks than cells at 37°C exposed to the same antibody (Fig. 3). These results suggest that heat shock is protecting the cells against the cytoskeletal perturbations induced by the thrombospondin antibody. One of us (N.V.K.) has recently shown that BAEC exposed to 43°C for 2–4 h show increased expression of several heat-shock proteins: namely, HSP71, 73, 80, 90 and 100 (Ketis et al. 1988a). Three of these are known to be present with cytoskeletal proteins: HSP73 (cognate) with microtubules (Lim et al. 1984), and HSP90 and -100 with actin (Koyasu et al. 1986). One important function of the HSP70 family appears to be ATP-dependent protein folding (Gasser and Shimke, 1986). Given that cells that are heat-stressed and allowed to recover in the presence of thrombospondin antibody at 37°C, synthesize stress proteins (Ketis et al. 1988a), show less retraction of cell processes, and more integrity of cytoskeletal structures than control cells exposed to antibody (Figs 4 versus 3), then it is easy to...
imagine that one or several of these heat-shock proteins could render the endothelial cells resistant to the effects elicited by the antibody.

It has been reported that in neuroblastoma cells exposed to heat stress, the microtubules reorganize and the intermediate filaments collapse about the nucleus (Wiegant et al. 1987). In contrast, in hepatoma cells destruction of stress fibres occurs, accompanied by rounding up of cells (Wiegant et al. 1987). Changes in the cytoskeletal organization of stress fibers (Glass et al. 1985), microtubules (Cross et al. 1982) and intermediate filaments (Thomas et al. 1982; Ketis et al. 1988b) as a result of heat stress has been reported for several mammalian cells. Alterations in cellular morphology have been described (Welch and Suhan, 1986). This suggests that heat-shock proteins are involved in the organization of the cytoskeletal networks during heat treatment and during recovery from heat treatment. Recently, Wiegant et al. (1987) reported that in hepatoma and neuroblastoma cells the HSPs are involved in the acquisition, maintenance and decay of thermotolerance (defined as heat resistance induced by heat itself) in the cytoskeleton.

In bovine aortic endothelial cells exposed to heat stress (Figs 1 and 2), loss of cytoskeletal integrity was observed for vimentin, actin and tubulin networks. During recovery from heat shock at 37°C, the cytoskeletal networks in endothelial cells showed distinct time-recovery (reorganization) characteristics. Specifically, during recovery at 37°C of cells from thermal insult, the vimentin cytoskeletal network reorganized to its apparent native form within 120 min, the actin network within 60 min and the tubulin network within 30 min as determined by immunofluorescence microscopy.

We have previously shown that cells exposed to hyperthermic challenge followed by a recovery period at 37°C, show an increase in mRNA levels for thrombospondin (Ketis et al. 1988b), and Welch and Suhan (1986) have shown that heat-stressed cells regain a normal morphology during later times of recovery. In addition, growth of endothelial cells after exposure to thermal insult is
initially retarded followed by exponential growth (N. V. Ketis, J. Lawler and M. J. Karnovsky, unpublished data). Thus we speculated that thrombospondin may be involved in the recovery of endothelial cells from a stressed or injured state (Ketis et al. 1988a). In this study, we show that the recovery of cells at 37°C from hyperthermia is inhibited by the presence of a specific antibody to human platelet thrombospondin, as reflected by the inability of these endothelial cells to reorganize their cytoskeleton to the form observed in control cells and by the antibody effect on cell spreading.

In summary, we present several novel observations, namely (1) that during recovery from thermal insult at 37°C, intermediate filaments, stress fibers and microtubules show distinct time-recovery (reorganization) characteristics in bovine aortic endothelial cells; (2) that in the presence of a monoclonal antibody to human platelet thrombospondin, the vimentin, actin and tubulin cytoskeletal networks of endothelial cells at 37°C are notably altered; (3) that the thrombospondin antibody used herein, which binds the thrombospondin heparin-binding domain, causes retraction of endothelial cell processes; (4) that endothelial cells exposed to hyperthermia followed by recovery at 37°C in the presence of thrombospondin antibody maintain the integrity of the cytoskeletal networks better than cells at 37°C exposed to thrombospondin antibody; and (5) that the recovery of the cytoskeleton in endothelial cells from hyperthermia is inhibited by this thrombospondin antibody. Our data suggest that the recovery of cells from heat shock requires the integrity of thrombospondin and its interactions. We speculate that thrombospondin may be involved in the recovery of endothelial cells from a stressed or injured state.

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