Effects of in vitro heat shock on immune cells in diet-induced obese mice

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ABSTRACT

Obesity has been associated with impaired immune responses and inflammation. The mechanisms underlying these immune disturbances in obesity are not yet clarified. This study investigated the effects of in vitro heat shock (HS) on immune cells from the point of view of thymocyte apoptosis and T-cell mitogen-stimulated splenocyte cytokine production as well as the heat shock protein 70 (HSP70) protein levels in diet-induced obese mice to explore a possible association between the disturbance of T cell immunity and HS response in obesity. Obese mice had increased apoptotic and necrotic thymocytes populations and increased splenocyte cytokine production of both proinflammatory and anti-inflammatory cytokines compared with lean mice. The in vitro HS at 42 °C decreased the rate of live cells in thymocytes, and the degree of the decrease was larger in obese mice compared with lean mice. The in vitro HS increased the intracellular and extracellular HSP70 protein levels in thymocytes and splenocytes, while the effects of obesity on the HSP70 protein levels were not obvious. The in vitro HS prior to T cell mitogen stimulation decreased IFN-γ and IL-10 production by mitogen-stimulated splenocytes. This change in cytokine production due to HS was not affected by obesity. The obvious alteration of the HSP70 protein levels and association between cytokine production and the HS response in obesity were not found in this obesity model; however, our results indicate an association between the viability of thymocytes and an altered HS response in obesity and provide evidence that the increase in thymocyte apoptosis and acceleration of thymus involution in obesity could be, in part, due to the alteration of the HS response.

1. Introduction

Obesity has become a worldwide epidemic (World Health Organization, 2016). The high incidence of inflammation-associated chronic disease and susceptibility to infection in obese people represent a growing health threat (Kanneganti and Dixit, 2012). Obesity-induced inflammation contributes to the development of several chronic conditions, including type 2 diabetes, atherosclerosis, and some forms of cancer (Kanneganti and Dixit, 2012). The impaired immune response in an obese host increases susceptibility to infection by various pathogens, leading to diseases such as influenza and community-acquired tuberculosis (Karlsson and Beck, 2010).

Accelerated thymic aging in obesity has been demonstrated in human studies and animal studies (Yoshida et al., 2014; Yang et al., 2009) as one of the mechanisms underlying the immunological complications of obesity. The thymus is the primary lymphoid organ for the development of T cells. Age-related involution of the thymus is associated with a decline in naïve T cell output, which contributes to a reduction in T cell diversity and leads to increased susceptibility to infection, autoimmune disease, and cancer (Palmer, 2013). A study in diet-induced obese mice demonstrated that accelerated age-related thymic involution in obesity is associated with defects, including increased apoptosis of developing thymocytes (Yang et al., 2009). Additionally, T cells in obesity are altered in number, subset distribution, and/or function to provide proinflammatory signals that support both systemic inflammation and adipose tissue inflammation (Ip et al., 2015). Our laboratory previously reported that cytokine production by splenocytes stimulated with T-cell mitogen is altered in diet-induced obese mice (Mito et al., 2000). Several obesity-associated changes, such as altered adipokine signaling and metabolic changes, are associated with disturbed immunity; however, the mechanisms underlying these immune alterations in obesity are not completely clear (Karlsson and Beck, 2010; Milner and Beck, 2012).

The heat shock (HS) response is a highly conserved primitive response that is essential for surviving against a wide range of stresses (Singh and Hasday, 2013). A characteristic feature of the HS response is...
rapid stress-induced synthesis of heat shock proteins (HSPs) (Morimoto and Santoro, 1998). In addition to their roles in cytoprotection, the HS response and HSPs have been found to play various roles in innate and adaptive immune responses (Muralidharan and Mandrekar, 2013), regulation of apoptosis (Joly et al., 2010), and regulation of cytokine production (Kappel et al., 1991; Hooper and Hooper, 2009; Stocki et al., 2012). In the context of obesity, recent studies have demonstrated that reduced HSP expression is associated with insulin resistance (Hooper and Hooper, 2009; Chung et al., 2008; Kurucz et al., 2002; Bruce et al., 2003; Kavanagh et al., 2009). Inducible HSP70 (also called HSP72) belongs to the HSP70 family and is a stress-induced and dominant member of the HSP family. HSP70 expression is decreased in the skeletal muscle of patients (Chung et al., 2008; Kurucz et al., 2002; Bruce et al., 2003) and in some organs in animal models (Chung et al., 2008; Kavanagh et al., 2009) with insulin resistance or type 2 diabetes. In addition, the HS-induced HSP70 levels in peripheral blood mononuclear cells are lower in type 1 diabetic subjects compared with non-diabetic subjects (Burkart et al., 2008). These studies have led us to speculate that obesity might alter the HS response, including HSP70 expression in immune cells, leading to disturbed immune function. In the present study, we investigated the effects of in vitro HS on thymocyte apoptosis and T-cell mitogen-stimulated splenocyte cytokine production as well as the HSP70 protein levels of thymocytes and splenocytes of obese mice to explore the possible association between the disturbance of T cell immunity and HS response in obesity.

2. Material and methods

2.1. Animals

Five-week-old male C57BL/6J mice were obtained from Sankyo Laboratories (Tokyo, Japan). Mice were allowed free access to water and food and were kept under a 12 h:12 h light:dark cycle in a temperature- and humidity-controlled environment. This study was approved by the Institutional Animal Care and Use Committee at Japan Women’s University, and animals were maintained in accordance with the Guidelines for Proper Conduct of Animal Experiments by the Science Council of Japan.

2.2. Experimental Procedures

Mice were fed a standard diet (AIN-93M; American Institute of Nutrition 93 M; 15.4% of energy as protein, 10.6% as fat, and 74.1% as carbohydrate) over an acclimation period of 1 week. At 6 weeks of age, mice were randomly divided into two groups: obesity-induced mice (n = 5), which were fed a high-fat diet (10.3% of energy as protein, 59.7% as fat, and 30.0% as carbohydrate) with the same composition as the AIN-93M diet, except that the diet was supplemented with 29% w/w lard instead of cornstarch, and lean mice (n = 5), which were fed the AIN-93M diet.

After 7 weeks of administration of experimental diets, 1 week before sacrifice, blood samples were collected from the tail vein of mice after overnight fasting to measure their blood glucose levels using an automatic glucose analyzer (Arkay Factory, Shiga, Japan) and plasma insulin concentration using a sandwich enzyme-linked immunosorbent assay (ELISA) with a Revis insulin kit (Shibayagi, Gunma, Japan). The homeostasis model assessment of insulin resistance (HOMA-IR) as an index of insulin resistance was calculated using the following formula: HOMA-IR = fasting insulin (μU/ml) × fasting glucose (mg/dl) / 405 (Saraswathi et al., 2009). After 8 weeks of experimental diet feeding, the body weights and fat pads were weighed. Then, both the thymus and spleen were aseptically removed for single cell suspensions in RPMI-1640 medium (Nissui, Tokyo, Japan) supplemented with 5% heat-inactivated fetal bovine serum (Cell Culture Bioscience, Nichirei Biosciences, Tokyo, Japan), 2 mM l-glutamine (Life technologies, CA, USA), 100 Units/ml penicillin and 100 μg/ml streptomycin (Life technologies), and 50 μM 2-mercaptoethanol (Wako, Osaka, Japan) and counted using a hemocytometer as described previously (Mito et al., 2000). The thymocytes and splenocytes were subjected to flow cytometric analysis, proliferative response analysis, and HS experiments.

2.3. Flow cytometric analysis

The thymocytes and splenocytes underwent flow cytometric analysis as described previously (Hamaguchi et al., 2012). Thymocytes were stained with anti-CD4-PE and anti-CD8-FITC (BD Biosciences, CA, USA). To analyse apoptosis, thymocytes were stained with anti-annexin V and propidium iodide (PI) using an annexin V-FITC apoptosis detection kit (BioVision, CA, USA). Splenocytes were stained with anti-CD3-FITC (BD Biosciences) to identify T cells, with anti-CD4-PE and anti-CD8-PE (BD Biosciences), as well as with anti-CD4-PerCP, anti-CD45RB-FITC, and anti-CD44-FITC (BD Biosciences) to identify naive and memory effector CD4+ T cells. Cells were analyzed by flow cytometry using a Gallios flow cytometer and Kaluza software (Beckman Coulter, CA, USA). The acquired data were analyzed after gating on lymphocytes based on forward and side scattering.

2.4. Proliferative response of splenocytes

Splenocytes (5 × 10⁴ cells/100 μl per well) were cultured in the presence of 5 μg/ml concanavalin A (ConA; Sigma, MO, USA) at 37 °C in a 5% CO₂ atmosphere for 48 h and, then, 10 μl/well of Alamar Blue solution (Bio-Rad, CA, USA) was added to the culture. After an additional incubation for 4 h, fluorescence was measured at 544/590 nm. The results are expressed as the stimulation index (SI), which is defined as the ratio of fluorescence from ConA-stimulated wells to fluorescence from non-stimulated wells.

2.5. Heat shock experiment

2.5.1. Thymocyte experiment

HS stimulations were performed by incubating the thymocyte cultures (5 × 10⁶ cells/500 μl per well) at 42 °C for half an hour in a 5% CO₂ atmosphere. Non-heat-shocked cells were used as the control and were incubated at 37 °C in a 5% CO₂ atmosphere.

After an additional overnight culture at 37 °C in a 5% CO₂ atmosphere, one-tenth of each culture of heat-shocked thymocytes and non-heat-shocked thymocytes were subjected to apoptosis analysis using the apoptosis detection kit as described in Section 2.3.

Heat-shocked thymocytes, non-heat-shocked thymocytes, and culture supernatants were collected by centrifugation of the remaining nine-tenths of each culture. The HSP70 concentration in the culture supernatant was measured by ELISA with Duoset kits (R & D Systems, MN, USA) to examine the quantity of HSP70 released into the extracellular environment. The intracellular HSP70 protein levels in the collected cells were determined as described in Section 2.5.3.

2.5.2. Splenocyte experiment

HS stimulations were performed by incubating the cultures of splenocytes (5 × 10⁶ cells/500 μl per well) at 42 °C for 1 h followed by recovery at 37 °C for 1 h in a 5% CO₂ atmosphere. Non-heat-shocked cells were used as the control and were incubated at 37 °C in a 5% CO₂ atmosphere for 2 h.

Heat-shocked splenocytes and non-heat-shocked splenocytes were additionally cultured with or without 5 μg/ml ConA at 37 °C in a 5% CO₂ atmosphere for 24 h. Cells and supernatants were collected by centrifugation. The concentration of HSP70 in the collected supernatants of the culture without ConA stimulation was measured by ELISA with Duoset kits (R & D Systems). The intracellular HSP70 protein levels in the collected cells, which were cultured without ConA stimulation, were measured as described in Section 2.5.3. The concentrations of interferon (IFN)-γ, interleukin (IL)-4, IL-17, and IL-10 in
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