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Chronic restraint stress modulates expression of genes in murine spleen

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Abstract

Psychological and physical stress can alter the immune system in both humans and animals. We have reported that mice subjected to chronic 12-h daily physical restraint for 2 days showed dramatic apoptosis in splenocytes. To identify genes that contribute to the splenocyte apoptosis, we compare gene expression in the spleens of restrained and unstressed mice using oligo microarrays consisting of 226 genes. We report here that mice subjected to chronic 12-h daily physical restraint for 2 days exhibited significantly altered expression of 50 of 226 genes. These genes included pro-apoptotic genes. We selected 5 genes of interest and confirmed the microarray results by real-time PCR. In this study, we identify a potentially important component of pro-apoptotic activity in restraint stress and suggest a possible target for anti-apoptotic therapy to protect splenocytes against stress-induced apoptosis.

Keywords: Restraint stress; Apoptosis; Mice; Gene expression; Microarray

1. Introduction

Stress affects our daily lives. Many studies have shown that stress has profound effects on immunological parameters in humans and animals (Frieri, 2003; Yang and Glaser, 2002; Padgett and Glaser, 2003), including lymphopenia, leukocyte subset distribution, cytokine production, natural killer cell activity, and macrophage maturation and activity. These changes may result from physical or psychological stress (Yang and Glaser, 2002; Sonnenfeld, 1998). It is generally believed that moderate stress, such as routine exercise, could enhance immune function, while chronic stress, such as long-term emotional stress, could decrease immune response and increase susceptibility to diseases (Dhabhar and McEwen, 1997), which is at least in part due to the reduction of lymphocytes (Berthiaume et al., 1999; Zorrilla et al., 2001). Stress-induced lymphopenia has been observed in persons under various kinds of psychological stress (Zakowski et al., 1992), such as surgical patients (Galinowski, 1993; Kunes and Krejsek, 2000).

Stress-induced changes in immune function may be due to the effects of neurohumoral factors that play a fundamental role in the psychoneuroimmune to stress which can produce changes in immune cells that can, in turn, activate apoptosis in other immune cells. For example, it has been reported that co-culture of T cells with monocytes from stressed patients induced apoptosis in the T cells, whereas co-culture with monocytes from unstressed patients did not (Kono et al., 2001). This observation may lead to the identification of cells responsible for providing a cell apoptotic signal to lymphocytes. Mice experiencing restraint

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stress demonstrated decreased concanavalin A (ConA)-driven lymphocyte proliferation, and natural killer (NK) cell activity in the spleen (Marotti et al., 1996). Apoptosis is a key regulator of tissue homeostasis (Debatin and Krammer, 2004). Apoptotic cell death is an active process mediated by various signaling pathways, which include the caspase cascade pathway. The caspase cascade is activated by two distinct routes: one from the cell surface and the other from mitochondria. Activation of the route from the cell surface requires cellular components that include membrane receptors, adaptor proteins such as Fas-associated death domain (FADD) and caspase-8. Activation of the pathway from mitochondria requires Apaf-1 and cytosolic cytochrome *c*.

Our previous studies (Yin et al., 2000) utilized the restraint stress mouse model and have shown that chronic restraint stress results in a reduction number of splenic lymphocytes through cell death receptor Fas-mediated apoptosis pathway in an endogenous opioid-dependent manner. However, the cellular machinery for apoptosis underlying the beneficial effects of genes on restraint stress remains to be elucidated. To determine which genes might be involved in the effects of stress DNA microarray technology, which has recently emerged as a powerful tool, allowed us to simultaneous analyze and compare genes expressed in normal and treated cells (Renaud et al., 2005; Duggan et al., 1999). We compared the gene expression pattern in the spleens of mice either exposed or not exposed to restraint stress. Using oligo microarrays containing 226 genes of various functional classes, we found that 49 genes were up-regulated whereas 1 gene was down-regulated. We selected 5 genes of particular interest and confirmed these genes by quantitative real-time PCR.

2. Materials and methods

2.1. Physical restraint

Six- to eight-week-old male Balb/c mice were obtained from Charles River Laboratories (Wilmington, MA) and maintained in the Division of Laboratory Animal Resources at East Tennessee State University (ETSU), a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The mice were subjected to an established chronic physical restraint protocol used in our laboratory as well as others (Yin et al., 2000; Sheridan et al., 1998). Briefly, mice were placed in a 50-ml conical centrifuge tube filled with multiple punctures to allow ventilation. Mice were held horizontally in the tubes for 12 h followed by a 12-h rest. During the rest period food and water were provided ad libitum. Control littermates were kept in their original cage and food and water were provided only during the 12 h of rest. This physical restraint procedure was approved by the Animal Care and Use Committee of ETSU. At 2 days after physical restraint, mice were sacrificed by CO_2 asphyxiation, and the spleens were rapidly frozen in liquid nitrogen and stored at −80 °C for isolation of total RNA.

2.2. Isolation of RNA from spleen tissue

Total RNA was isolated from spleens using the VERSAGENE™ RNA Tissue Kit (Gentra SYSTEMS; Minnesota) according to the manufacturer's instructions. DNAse I treatment (Qiagen, Chatsworth, CA) was applied to eliminate genomic DNA interference (Wang et al., 1998). RNA quality was determined by running a sample with a RNA loading dye (Sigma–Aldrich) on a 1% agarose gel and inspecting for distinct 18S and 28S bands, indicating lack of degradation. Quantity was determined by A_{260} measurement (Yin et al., 1999, 2000). Samples were frozen at −80 °C until use in microarray experiments.

2.3. Analysis of gene expression using oligo gene microarrays

The methodology for microarray analysis has been described in elsewhere (Ghaffari et al., 2006; Han et al., 2005). Specific gene expression oligo GEArray systems were purchased from SuperArray Bioscience Corporation (Frederick, MD, USA). Each oligo GEArray mouse apoptosis (catalog number: OMM-012) and p53 signaling pathway (catalog number: OMM-027) array consists of 113 genes known to be involved in apoptosis and p53-mediated signaling. Using different arrays, we compared the gene expressions in spleen tissues from mice with or without restraint stress. The microarrays were used according to the manufacturer's instructions. Briefly, using the reagents provided, the RNA was enzymatically converted to biotinylated cRNA target with the TrueLabeling-AMP™ 2.0 Kit in a standard thermal cycler. The resulting biotinylated cRNA probe mixture was allowed to hybridize overnight to the GEArray microarrays in a HybTube in a standard hybridization oven at 60 °C. After washing two times and blocking array membranes for 40 min at room temperature, alkaline phosphatase-conjugated streptavidin was allowed to bind and then CDP-Start substrate chemiluminescence was detected by exposure to a Chemi-Doc imaging system (CCD camera). Loading was adjusted based on the intensity of hybridization signals to the housekeeping gene, and then gene expression was quantified by scanning densitometry. Data were acquired and analyzed with the web-based software package GEArray ^{R} Expression Analysis Suite provided by SuperArray Bioscience Corporation (Frederick, MD).

2.4. Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR)

The real-time PCR detection technique was performed as described recently (Beersma et al., 2005). Briefly, first-strand cDNA was synthesized from 1 μg of total RNA in a final volume 50 μl using a Reaction Ready™ first strand cDNA synthesis kit (SuperArray Bioscience Corporation, Frederick, MD). After incubation at 70 °C for 3 min and cooling down to 37 °C for 10 min, RT cocktail was added to the annealing