Brief Definitive Report

Chronic Restraint Stress Promotes Lymphocyte Apoptosis by Modulating CD95 Expression

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Abstract

Depending on the duration and severity, psychological tension and physical stress can enhance or suppress the immune system in both humans and animals. Although it is well established that stress alters the release of various hormones and neurotransmitters, the mechanisms by which stress affects immune responses remain elusive. We report here that mice subjected to chronic 12-hour daily physical restraint for two days exhibited a significant reduction in splenocytes, a process likely mediated by apoptosis as demonstrated by the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling assay. CD95 (Fas/APO-1) expression in splenic lymphocytes of stressed mice was substantially increased. Interestingly, Fas-immunoglobulin fusion protein and blocking antibodies against CD95 ligand inhibit stress-induced reduction in lymphocytes. The stress-induced changes in CD95 expression and lymphocyte number could be blocked by naltrexone or naloxone, specific opioid receptor antagonists, indicating a pivotal role of endogenous opioids in this process. In addition, the reduction of splenocytes in this model system seems to be independent of the hypothalamo-pituitary-adrenal axis, as both adrenalectomized and sham-operated mice exhibited similar responses to chronic stress. Moreover, chronic physical restraint failed to induce a decrease in lymphocyte numbers in CD95-deficient (Fas−/−) mice. Therefore, stress modulates the immune system through CD95-mediated apoptosis dependent on endogenous opioids.

Key words: stress • fas antigen • lymphocyte • apoptosis • endogenous opioid

Introduction

Bidirectional interactions between the immune and neuroendocrine systems influence antibody and cytokine responses (1, 2), cytolytic activity, lymphocyte proliferation (3), tissue localization and number of lymphocytes (4, 5), hypothalamic-pituitary hormone secretion (6), and neural signal transmission (7–9). These interactions are likely involved in the maintenance of cellular homeostasis in several systems, which is best exemplified in response to stress. Recent progress in psychoneuroimmunology has revealed that stress could either suppress or enhance immune responses depending on the type and duration of the stressors (10–13). Numerous studies have revealed that exhausting physical activity and severe environmental and/or psychological stress have strong suppressive effects on the immune system (14). Such suppression of the immune system has significant implications for disease susceptibility and progression. Investigations in both humans and animals have revealed that stress could promote tumor development (15, 16), autoimmunity (17), and infectious diseases by influencing the onset, course, and outcome of the pathological processes (18, 19). Interestingly, acute psychological stressors and moderate physical exercise transiently enhance immune responses (10, 11, 20–23). In a rodent model, Dhabhar and McEwen (13) recently demonstrated that acute restraint stress (2 h) could dramatically enhance delayed-type hypersensitivity reaction. In addition, acute stress has also been shown to increase antibody production (24). Though various changes in the immune system have been shown to be associated with stress, the exact mechanisms responsible for stress-modulated immune response remain to be elucidated.

CD95 (also known as Apo-1 or Fas), a transmembrane protein belonging to the tumor necrosis factor/nerve
growth factor receptor family of cell surface molecules, is expressed on a variety of cell types including lymphocytes, hepatocytes, ovarian epithelial cells, and some tumor cells (25). Ligation of this molecule with specific agonistic antibodies or its cognate ligand (CD 95L) induces the activation of a cascade of caspases and ultimately nucleases that result in apoptotic cell death in many cell lineages (26, 27). CD 95 was initially identified in the immune system, and has been shown to mediate receptor-dependent programmed cell death. Although the importance of Fas–FasL interactions is not limited to the immune system, it is there that most of the functional studies of Fas–FasL have originated. It is well documented that activation-induced apoptosis in peripheral T cells and T cell hybridomas is mediated through Fas–FasL interaction and is believed to serve as a guard against autoimmunity (26, 28, 29). Mice and humans with mutations in CD 95 or CD 95L develop lymphocyte accumulation disease (30). Thus, the CD 95–CD 95L system plays an integral role in maintaining cellular homeostasis of the immune system and may contribute to the alteration of the immune system under chronic stress.

Stress induces the production of various hormones and neural peptides (22, 31). Corticosteroids and endogenous opioids are the best-recognized mediators modulating the immune response. It is well established that corticosteroid plays a critical role under stress conditions (32). Corticosteroid enhances immune response during acute stress (13, 21) and suppresses the immune response during chronic stress (13). On the other hand, endogenous opiate peptides are known to be elevated by both acute and chronic stress, and play a critical role in regulating behavioral and emotional stress-induced changes of the immune system (33). These peptides could induce the production of corticosterone via the hypothalamo-pituitary-adrenal (HPA) axis (34). T cells with high expression of opioid receptors have been identified, all of which have been found on lymphocytes (35). Blockade of endogenous opioids with naloxone has been demonstrated to attenuate or reverse stress-induced elevation in blood pressure. Recent studies have revealed that excessive production of endogenous opioids under situations of chronic stress is associated with the suppression of the immune system (36). Because exogenous opioid receptor agonists such as morphine induce a reduction in the number of lymphocytes by modulating CD 95 expression (37), we examined the role of CD 95 in the modulation of the immune system by chronic stress. We show in this study that chronic 12-h daily physical restraint for 2 d promotes CD 95 expression dependent on endogenous opioid, and that the subsequent induction of apoptosis through CD 95 plays a central role in stress-induced reduction of lymphocytes.

Materials and Methods

Physical Restraint. 7-9-wk-old mice were subjected to an established chronic physical restraint protocol (19). They were placed in a 50-ml conical centrifuge tube filled with multiple punctures to allow ventilation. Mice were held horizontally in the tubes for a continuous 12 h followed by a 12-h rest, while food and water were provided ad libitum. The control littersmates were kept in their original cage, but food and water were provided only during the stress interval of the experimental groups. Mice were physically restrained for one to three cycles as specified. This physical restraint procedure was approved by the Institutional Animal Care and Use Committee of the Holland Laboratory of the American Red Cross.

A analysis of CD 95 Expression. Total RNA was isolated from spleens by Trilu™ Isolation Reagent (Boehringer Mannheim) according to a protocol recommended by the manufacturer. RNA samples were fractionated on 1% agarose/2.2 M formaldehyde denaturing gel and transferred onto Nytran™ membrane (Schleicher & Schuell). The cDNA probes (CD 95 and glyceraldehyde-3-phosphate dehydrogenase [GAPDH]) were labeled with 32P-dCTP by random priming method (Boehringer Mannheim) according to the manufacturer's instructions. Hybridization signals were detected by autoradiography.

DNA Fragmentation. Nucleosomal DNA fragmentation in spleens was determined by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay using an in situ apoptosis detection kit (Trevigen) according to the manufacturer's instructions. The 3’-OH ends of fragmented nucleosomal DNA were specifically labeled in situ in the presence of exogenously added terminal transferase and fluorescein isothiocyanate-labeled dUTP, and were detected with anti-fluorescein antibody peroxidase conjugate.

Genomic DNA integrity also was determined by DNA content in the nuclei. Cells were fixed with 70% ethanol for 30 min at 4°C, then washed twice with PBS. The fixed cells were then incubated in PBS containing propidium iodide (Sigma Chemical Co.) at 50 μg/ml and RNaseA (Boehringer Mannheim) at 0.1 mg/ml at room temperature for 30 min. DNA content was determined by flow cytometry, and the FL2 intensity was plotted as a histogram on a linear scale.

Induction of Apoptosis by CD 95 Ligation. Single cell suspensions of murine splenocytes were prepared by gently squeezing the spleen between frosted glass microscope slides. Red blood cells were lysed with buffered 1% ammonium chloride. Splenocytes were then cocultured with L cells expressing sense or antisense CD 95L (provided by Dr. T.A. Ferguson, Washington University School of Medicine, St. Louis, MO). Apoptosis in nonadherent cells was determined at 24 h by flow cytometric DNA content analysis.

Adrenalectomy. Bilateral adrenalectomy was performed on 10-wk-old Balb/c mice. Mice were anesthetized by intraperitoneal injection of ketamine (90 mg/kg) plus xylazine (10 mg/kg). A 0.5-cm skin incision was made on the back. The skin on both sides of the incision was side moved, and a muscle incision was made on the top of each adrenal gland. The entire adrenal gland was removed with a pair of heated fine forceps. The skin incision was closed with a surgical clip. After recovery, the mice were maintained by providing food, water, and sodium chloride block ad libitum. Control animals (sham group) underwent the same surgical procedure as the adrenalectomized animals, except their adrenal glands were not removed. Experiments were performed with these mice at 2-3 wk after adrenalectomy.

Results and Discussion

We subjected BALB/c mice aged 7-9 wk to a 12-h physical restraint regimen daily for 2 d (19, 38) and found
that this treatment dramatically affected splenic cellularity. These mice showed 35–40% reduction in their number of lymphocytes in the spleen compared with unstressed controls (Fig. 1 A). Since it is well established that overproduction of endogenous opioids could dramatically affect the immune system, we employed naltrexone or naloxone as specific antagonists of opioid receptors to determine the role of endogenous opioids in the reduction of splenic lymphocytes in this chronic stress model. Although treatment of mice with opioid antagonists (single intraperitoneal injection 1 h before the initiation of each stress cycle) did not alter the number of splenocytes in unstressed mice, administration of naltrexone (Fig. 1 A, experiment [Exp.] 1) or naloxone (data not shown) before physical restraint completely blocked stress-induced reduction in splenocyte numbers. Therefore, chronic physical restraint–induced lymphocyte reduction appears to require endogenous opioids.

We recently reported that exogenous opioids could induce CD95 expression and promote CD95L-induced apoptosis in lymphocytes in both in vitro culture and in vivo administration to mice (37). To verify the role of CD95 in chronic stress–induced lymphocyte reduction, we injected mice with serum from SCID mice bearing either 3T3 fibrosarcoma engineered to secrete Fas-Ig fusion protein (39) or nonengineered 3T3 fibrosarcoma. Fas-Ig–containing serum blocked chronic restraint stress–induced reduction in splenocytes (Fig. 1 A, Exp. 2), whereas normal mouse serum did not exhibit such an effect (data not shown). When the Fas-Ig–containing serum was adsorbed with protein A sepharose beads, its protective effect was eliminated (data not shown). In addition, the Fas-Ig serum did not change the number of splenocytes in unstressed mice. We also found that a blocking antibody to CD95L (MFL3) but not the isotype control also blocked stress–induced reduction in lymphocyte numbers (Fig. 1 A, Exp. 3). Hence, the interaction between CD95 and CD95L is critical for chronic stress–induced reduction in lymphocytes.

We next examined the level of CD95 in splenocytes with or without physical restraint. As shown in Fig. 1 B, the expression of CD95 in splenocytes was significantly enhanced at 12 h after physical restraint as detected by Northern blot analysis. Stress did not induce the expression of CD95L (data not shown). Naltrexone inhibited this stress–induced increase in CD95 expression. Interestingly, though MFL3 prevented stress–induced reduction in lymphocytes, it did not affect stress–induced CD95 expression (Fig. 1 B). The effect of stress on CD95 expression was also observed when detected by surface staining (data not shown). These results suggested a prominent role of endogenous opioids in the induction of CD95 expression.

The reduction of splenocytes induced by stress could be mediated by two possible mechanisms: emigration or cell death. First, we assessed cell death in histological sections of spleens using a TUNEL technique. We found that a significant number of cells in the spleen of stressed mice were undergoing apoptosis, whereas only a few apoptotic cells were detected in the spleen of control mice (Fig. 2 A). Thus, physical restraint–induced reduction in splenocyte numbers is likely due to the induction of apoptosis rather than the mobilization of lymphocytes. An effect of blocking CD95L with MFL3 (anti-CD95L) on apoptosis was also observed when examined by TUNEL (Fig. 2 A). Therefore, stress–induced CD95 expression appears responsible for the lymphocyte loss.

To examine whether stress–induced CD95 expression modulates the sensitivity of lymphocytes to CD95L–mediated apoptosis, we isolated splenocytes at the end of the 12-h physical restraint and cocultured with adherent L cells transfected with either sense or antisense CD95L cDNA for 24 h. Splenocytes in suspension were harvested, and cell viability was analyzed by cellular DNA content analysis. As shown in Fig. 2 B, splenocytes from chronically stressed mice underwent apoptosis as indicated by the appearance of a hypodiploid peak upon incubation with L cells expressing sense CD95L, whereas control cells showed only minimal apoptosis. This stress–induced CD95–mediated apoptosis is specific, as cells from stressed mice did not undergo apoptosis when cocultured with L cells transfected
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with antisense CD95L cDNA. Thus, chronic stress-induced CD95 expression could promote CD95L-induced apoptosis.

Loss-of-function mutations in CD95 reduce apoptosis and cause the development of lymphocyte accumulation diseases in both humans and mice (27). To further investigate the role of CD95 in chronic physical restraint stress-induced reduction in lymphocyte numbers, we subjected mice bearing autosomal recessive mutations in CD95 (C3H.MRL.Fas<sup>lpr/lpr</sup>) and their appropriate background control, C3H/H<sub>ej</sub>, to physical restraint. Stress-induced reduction in the numbers of splenocytes was observed in C3H/H<sub>ej</sub> but not in C3H.MRL.Fas<sup>lpr/lpr</sup> mice (Fig. 3).

Therefore, mice with a loss-of-function mutation of CD95 lose their sensitivity to stress-induced reduction in lymphocyte numbers, further supporting the conspicuous role of CD95 for the stress response.

The blockade of chronic stress-induced lymphocyte reduction by opioid antagonists (Fig. 1 A) strongly suggests a pivotal role of endogenous opioids in this process. As it has been shown that opioids could exert their effects by modulating the production of corticosteroid via the HPA axis (34), we performed chronic restraint stress in adrenalectomized mice. We have found that adrenalectomy did not significantly affect the stress-induced reduction in splenocytes (Fig. 4). Therefore, the H PA axis is unlikely to be in-

![Figure 2](image-url)
involved in mediating the reduction of splenocytes in this chronic restraint stress model. Our finding of the absence of an effect of adrenalectomy on chronic stress–induced splenocyte reduction is in contrast with the demonstration of the role of the adrenal gland in acute stress–induced enhancement of delayed-type hypersensitivity response (21). This discrepancy is likely to be due to the stress duration and the evaluation parameters. Indeed, McEwen et al. (22) have suggested that the spleen is a relatively privileged site and is relatively inaccessible to endogenously produced corticosteroids. Therefore, our observation of the dispensability of adrenal glands in chronic stress–induced splenocyte reduction strongly suggests that the effects of endogenous opioids are likely exerted directly on splenocytes. This agrees with our previous finding that opioids induce the expression of Fas and sensitize cells to FasL–mediated apoptosis in cultured lymphocytes (37).

CD95 and its cognate ligand, CD95L, play a critical role in the regulation of cellular homeostasis, especially in the immune system. Loss-of-function mutations in the CD95 (lpr) and CD95L (gld) genes are characterized by excessive lymphoproliferation with accelerated autoimmune disorders (14). In addition, the interaction between CD95 and CD95L has also been shown to be important in maintaining immune-privileged sites such as Sertoli cells of the testis, the corneal epithelium, and the retina of the eye (27, 28). Interestingly, some tumors also express FasL, and this has been suggested as a mechanism to escape immune surveillance. Our finding of the role of CD95 in chronic stress–induced lymphocyte reduction reveals another important function of this molecule.

Stress affects our daily life. It is clear that moderate stress such as routine exercise could enhance immune response (11, 13). However, chronic stress such as long-term emotional stress can decrease immune function and increase disease susceptibility. Our demonstration of a central role for endogenous opioids and its link to cell death receptor CD95 expression in response to chronic restraint stress is of particular interest. We believe that identification of the specific opioid peptides and opioid receptor types involved in this process will provide biochemical understanding of the mechanisms by which the neuronal and the immune system communicate.

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