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Glucocorticoids regulate natural killer cell function epigenetically

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ABSTRACT

Although glucocorticoids are well known for their capacity to suppress the immune response, glucocorticoids can also promote immune responsiveness. It was the purpose of this investigation to evaluate the molecular basis for this apparent dichotomous immunologic effect. Glucocorticoid treatment of natural killer cells (NK) was shown to reduce NK cell cytolytic activity by reduction of histone promoter acetylation for perforin and granzyme B, which corresponded with reduced mRNA and protein for each. In contrast, glucocorticoid treatment increased histone acetylation at regulatory regions for interferon gamma and IL-6, as well as chromatin accessibility for each. This increase in histone acetylation was associated with increased proinflammatory cytokine mRNA and protein production upon cellular stimulation. These immunologic effects were evident at the level of the individual cell and demonstrate glucocorticoids to epigenetically reduce NK cell cytolytic activity while at the same time to prime NK cells for proinflammatory cytokine production.

1. Introduction

Glucocorticoids (GC) are primary stress hormones necessary for life that are synthesized in the adrenal cortex and released into the blood stream homeostatically and in response to environmental and physiological stress. GC orchestrate various physiological processes (e.g. metabolism, energy production, vascular tone, bone mineralization, central nervous system and immune function) by regulation of gene transcription, both induction and repression, of nearly 25% of the genome [1–3]. Because of their potent anti-inflammatory and immunosuppressive effects, synthetic GC are widely used to treat inflammatory and autoimmune diseases, and are known to suppress both innate and adaptive immune responses. GC suppress cell mediated lytic activities [4,5] as well as cytokine production [6–11] by many immune cell populations; including NK [4,12], T lymphocyte [13], monocyte [11,14,15] and macrophage [16]. Of particular note, natural killer cells and NK cell cytolytic activity (NKCA) are especially responsive to GC [4,12,17–19]. Even though GC are known to suppress immune function under some conditions, GC under other conditions can promote immune responsiveness [20–24]. Physiological doses of GCs have been shown to enhance immunoglobulin production by mitogen-stimulated human lymphocytes [25] and GC have been shown to stimulate B lymphocyte number and antibody production *in vitro* and *in vivo* [26]. Further, during the early stages of T-cell activation, low levels of GC enhance T-cell receptor induced lymphocyte proliferation increase T-cell responsiveness to IL-2 and enhance proliferation of memory T cells [27,28]. GC have been shown to synergistically enhance the induction of IL-1 beta and IL-6 [29] and the biological effects of IL-2, interferon (IFN) gamma, granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor, and oncostatin M [30]. These immune modulating effects of GC are concentration and time dependent [31,32] and it is clear that in addition to well-known immunosuppressive effects, GC are also able to exert modulating and enhancing effects upon the immune system [33–35]. In experimental models, both suppressive and immune enhancing effects of GC have been demonstrated experimentally for inflammatory cytokine mRNA and protein production by monocytes [11,14,15], phagocytosis by macrophages [16], acute-phase protein gene expression by hepatocytes [36], delayed-type hypersensitivity reactions [37] and wound healing [38]. In those studies, immune enhancing effects were observed at lower GC concentration and immune suppressive effects at higher GC concentration.

The timing of GC administration also affects immune outcomes in that a 24 h pre-treatment of experimental animals with GC potentiated the proinflammatory response to subsequent endotoxin challenge; whereas, the administration of GC 1 h after endotoxin challenge resulted in suppression of the proinflammatory response [20]. In human volunteers, a 2 week administration of

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dexamethasone (a synthetic GC) resulted in an attenuation of GC mediated inhibition of IL-6 and TNF-alpha production *in vitro* [39]. Further, experimentally increased plasma cortisol levels (concentrations of 75–85 μg/dL for a 6-h period) ending 12–144 h before injection of endotoxin, resulted in an increased proinflammatory response to the bacterial product [13]. In that study, time as well as GC concentration were important in determining the effect of GC. More recent work has demonstrated exposure of humans to cortisol concentrations of 35–45 μg/dL total plasma cortisol (approximately 80 nM free cortisol) enhanced inflammatory responses to a subsequent, stimulus with endotoxin [40]. Those results demonstrated a "preparative" or "priming" effect of GC on the immune proinflammatory response. A GC concentration of 35–45 μg/dL is similar to concentrations commonly observed during human systemic stress responses [40].

The basis for these effects of GC are not well understood, although various possibilities have been proposed [41–44] including; mechanisms upstream of the binding of GC to its receptor, modified intracellular GC concentrations or insufficient GR expression. Those studies also reported mechanisms downstream of the binding of GC to GR that involve GC signaling pathways [45,46]. In addition to these, another possibility is that GC influence epigenetic processes that result in the observed immunological effects. Histone tail post translational modifications (e.g. acetylation, methylation) regulate gene transcription [47–49] and GC have been shown to modify NK cell function epigenetically [50,51]. In those reports, GC at a high concentration reduced NKCA; global histone acetylation, the acetylation of histone (H) 4 lysine (K) position 8, and promoter accessibility for perforin, interferon gamma and granzyme B. These epigenetics effects corresponded with reduced production of granule constituents (perforin and granzyme B) as well as reduced constitutive and stimulated production of IL-6, TNF alpha and IFN gamma. Histone acetylation was fully recovered by treatment of the NK cells with a histone deacetylase inhibitor, which also restored NKCA and proinflammatory cytokine production levels. Those results demonstrated GC to dysregulate NK cell function through an epigenetic mechanism that reduced histone tail acetylation status, immune effector gene transcription and levels of immune effector proteins necessary to the full functional activity of NK cells [50]. Those results are consistent with the known immune suppressive effects of a high GC concentration on NK cell effector function. In the experiments described herein, the effect of a low GC concentration on NK cells was evaluated with the hypothesis that a dichotomous phenotype would be revealed by treatment of NK cells with a lower GC concentration.

2. Materials and methods

2.1. Cell culture

IL-2 dependent NK92 cells (established from a patient with non-Hodgkin's lymphoma with the capacity to lyse a broad range of leukemia, lymphoma and myeloma cell lines at low effector to target ratio *in vitro*) was obtained from the American Type Culture Collection, Rockville, MD and maintained in alpha MEM with 12.5% horse serum (Gibco Laboratories, Grand Island, NY), 12.5% fetal bovine serum (FBS) (Gibco Laboratories, Grand Island, NY), penicillin, streptomycin (Whittaker M. A. Bioproducts, Walkersville, MD), 0.2 mM inositol: (Sigma Aldrich, St. Louis, MO), 0.1 mM 2-mercaptoethanol; (Gibco Laboratories, Grand Island, NY) and 0.02 mM folic acid: (Sigma Aldrich, St. Louis, MO). NK92 cell cultures were also supplemented with IL-2 (100 units/ml). The human erythroleukemic like cell line, K562, was obtained from the American Type Culture Collection, Rockville, MD. K562 cells were maintained in suspension in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS (Gibco Laboratories, Grand Island, NY), 100 units/ml penicillin, 100 μg/ml streptomycin (Whittaker M. A. Bioproducts, Walkersville, MD), 0.1 mM non-essential amino acids and 2 mM L-glutamine (Gibco Laboratories, Grand Island, NY).

2.2. Cellular treatment

NK92s, cultured at 2.5×105 cells/ml, were treated with or without dexamethasone (GC) (Sigma Aldrich, St. Louis, MO) (10–10 M) for 4 days in the presence of IL-2 (100 units/ml), every 48 h cells were collected, washed, and resuspended in fresh media to a concentration of $2.5x10^5$ cells/ml supplemented with or without dexamethasone and IL-2 (100 units/ml). For the final 24 h of treatment cells were collected, washed, and resuspended to a concentration of 2.5×10^5 cells/ml with or without dexamethasone in the absence of IL-2. Following this treatment NK92s were washed with media and resuspended to 1×10^6 cells/ml and used for analysis. In all cases, cell number and viability were determined by exclusion of 0.1% Trypan blue. Viability was maintained between 85% and 95% in all cases. In some experiments, cell cultures described above were subjected to an additional 24 h treatment in the presence of GC (10^{-7} M) to determine the sensitivity of the cells to further GC treatment (switch to −7 M).

2.3. Evaluation of cytokine production

Cytokines were measured in NK92 culture supernatants as described previously [50]. To assess stimulated cytokine production, NK92s (2.5×10^5 cells/ml) were treated as described above and then stimulated with IL-2 (5000 U) or IL-12 (40 pg/ml) for an additional 4 h. Cell culture supernatants were harvested and aliquots of the supernatants were stored at −80 °C for subsequent cytokine determination by ELISA (R&D Systems, Minneapolis, MN).

2.4. Analysis of gene expression

Messenger RNA from NK92 cells was obtained using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). RevertAid First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON) was used to generate cDNA for quantitative real-time PCR from 500 ng total RNA. Negative reverse transcriptase samples confirmed absence of contaminating DNA. All reactions were performed in triplicate using SYBRgreen supermix (Biorad) with the following synthetic oligonucleotide primer pairs. IFN-gamma: forward 5′-TGG AAA GAG GAG AGT GAC AG-3′ and reverse 5′-ATT CAT GTC TTC CTT GAT GG-3′. IL-6: forward 5′-CAA CCT GAA CCT TCC AAA GAT G-3′ and reverse 5′-ACC TCA AAC TCC AAA AGA CCA G-3′. Perforin: forward 5′-GGA GTG CCG CTT CTA CAG-3′ and reverse 5′-CGT AGT TGG AGA TAA GCC TGA G-3′. Granzyme B: forward 5′-AAG ACG ACT TCG TGC TGA CA-3′ and reverse 5′-CCC CAA GGT GAC ATT TAT GG-3'. PCR cycles were denaturation at 94 °C (30 s) and annealing at 55–61 °C (30 s), no extension phase was necessary and performed using the Opticon 2 Real-Time Detector (Bio-Rad, Hercules, CA) and analyzed using the Opticon Monitor Software (Bio-Rad, Hercules, CA). The ΔΔC(t) method was used to determine changes in transcript levels between untreated and chronic GC treated cells using beta-actin as a reference gene.

$$\text{Fold change} = \frac{\text{Efficiency(target)} \wedge (\Delta C(t) \text{ target (control-treatment)})}{\text{Efficiency(reference)} \wedge (\Delta C(t) \text{ reference (control-treatment)})}$$

2.5. Natural killer cell cytolytic activity (NKCA)

K562 tumor cells were radioactively labeled with 100 uCi of [^{51}Cr] (New England Nuclear, Boston, MA). Radiolabeled K562 cells