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Oxidant-Mediated Mitochondrial Injury in Eosinophil Apoptosis: Enhancement by Glucocorticoids and Inhibition by Granulocyte-Macrophage Colony-Stimulating Factor

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The mainstay of asthma therapy, glucocorticosteroids (GCs) have among their therapeutic effects the inhibition of inflammatory cytokine production and induction of eosinophil apoptosis. In the absence of prosurvival cytokines (e.g., GM-CSF), eosinophils appear to be short-lived, undergoing apoptosis over 96 h in vitro. In a dose-dependent manner, GC further enhances apoptosis, while prosurvival cytokines inhibit apoptosis and antagonize the effect of GC. The mechanisms of eosinophil apoptosis, its enhancement by GC, and antagonism of GC by GM-CSF are not well-understood. As demonstrated in this study, baseline apoptosis of eosinophils resulted from oxidant-mediated mitochondrial injury that was significantly enhanced by GC. Mitochondrial injury was detected by early and progressive loss of mitochondrial membrane potential and the antioxidant protein, Mn superoxide dismutase (SOD). Also observed was the activation/translocation of the proapoptotic protein, Bax, to mitochondria. Underscoring the role of oxidants was the inhibition of mitochondrial changes and apoptosis with culture in hypoxia, or pretreatment with a flavoprotein inhibitor or a SOD mimic. GCs demonstrated early (40 min) and late (16 h) activation of proapoptotic c-Jun NH2-terminal kinase (JNK) and decreased the antiapoptotic protein X-linked inhibitor of apoptosis, a recently demonstrated inhibitor of JNK activation. Similarly, inhibition of JNK prevented GC-enhanced mitochondrial injury and apoptosis. Importantly, GM-CSF prevented GC-induced loss of X-linked inhibitor of apoptosis protein, late activation of JNK, and mitochondrial injury even in the face of unchanged oxidant production, loss of MnSOD, and early JNK activation. These data demonstrate that oxidant-induced mitochondrial injury is pivotal in eosinophil apoptosis, and is enhanced by GC-induced prolonged JNK activation that is in turn inhibited by GM-CSF. The Journal of Immunology, 2003, 170: 556–566.

Eosinophils are considered the primary effector cells of atopic diseases such as asthma and allergic rhinitis where there is evidence for their enhanced differentiation and release from bone marrow, selective adherence and chemotaxis, and prolonged survival (1). Ample evidence demonstrates up-regulation of the hemopoietic growth/survival factors (HGSFs), IL-5, GM-CSF, and IL-3 in these inflammatory diseases which direct the differentiation of eosinophils, but also prime and enhance their effector function (1). Indeed, eosinophils themselves contribute to the expression of these cytokines, particularly GM-CSF (references in Ref. 2), which may act in an autocrine/paracrine fashion. Once attracted into the asthmatic airway, eosinophils generate eicosanoids, cytokines, and reactive oxygen and nitrogen species, and most importantly, release highly cationic proteins (major basic protein, eosinophil cationic protein, eosinophil peroxidase), thought to inflict much of the damage to the asthmatic airway epithelium and submucosa (3).

Additionally, the HGSFs potently promote eosinophil survival. Blood eosinophils from asthmatic and atopic subjects demonstrate prolonged survival relative to eosinophils from normal control subjects (4, 5), and neutralizing Ab to IL-5 or GM-CSF induce apoptosis in polyp explants (6). Importantly, the number of apoptotic eosinophils and the ratio of apoptotic to total eosinophils has been inversely correlated with clinical severity of asthma (7). Additionally, tissue production of GM-CSF correlated with both the frequency of nonapoptotic eosinophils and the severity of asthma. These findings support the hypothesis that eosinophil longevity contributes to eosinophil accumulation in tissues, which in turn, correlates with disease severity (6–8). Recent studies show that the HGSFs protect eosinophils from apoptosis by inhibiting Bax translocation to mitochondria and subsequent mitochondrial injury with release of cytochrome c and induction of apoptosis (9, 10).

Glucocorticosteroids (GC), delivered either topically or systemically, are unequivocally the most potent therapeutic agents in the treatment of these eosinophilic conditions. They have protean effects on both inflammatory and resident cells and potently down-regulate the expression of IL-3, IL-5, and GM-CSF. GC treatment has been shown to induce eosinophil apoptosis both in vitro as well as in vivo (11–13). As systemic GC was administered to subjects with unstable asthma, the number of sputum eosinophils decreased, while the proportion of apoptotic eosinophils in sputum increased from 10% pretreatment to 70% posttreatment (14). Similarly, Druilhe et al. (15) biopsied relatively mild asthmatics and found a similar trend with only 2% of airway tissue eosinophils demonstrating apoptotic features in subjects not receiving GC vs 22% with GC treatment.

Lymphocytes, particularly thymocytes (16, 17), but also mature T cells (18, 19), are also known to undergo GC-induced apoptosis.
This appears to involve injury to mitochondria (17, 19), production of reactive oxygen species (ROS) (19–22), lipid peroxidation (23), and caspase activation. Importantly, GC-induced mitochondrial injury was shown to precede other apoptotic changes (17, 19). Notably, GC-induced mitochondrial injury and subsequent apoptosis was inhibited by the (over)expression of members of the antiapoptotic Bcl-2 protein family (23, 24), but not the addition of an inhibitor of downstream caspase-3 family proteases (25), suggesting that injury to mitochondria and subsequent release of cytochrome c and other proapoptotic proteins was pivotal.

By comparison, the mechanism by which GCs induce eosinophil apoptosis is poorly understood. To date, there is no evidence that GC alters expression of either the proapoptotic Bcl-2 proteins or the balance of these with antiapoptotic Bcl-2 proteins in eosinophils (12). The activation of caspases 2, 3, 6, and 8 has been demonstrated in eosinophil apoptosis by several groups, but enhanced activation due to GC treatment has not been generally documented until 24–48 h of culture (12, 13, 26), and inhibition of caspase 3 only marginally inhibits GC-induced apoptosis (27). Of interest, Arat et al. (26) found that the flavoprotein inhibitor, diphenylethenone (DPI) inhibited caspase 3-like activity and eosinophil apoptosis at 48 h of culture, suggesting caspase activation was downstream of ROS production. Based on these few studies and parallels with lymphocytes, we hypothesized that oxidant-induced mitochondrial injury would be an early event, pivotal to GC-induced eosinophil apoptosis. In this study, we show that in the absence of prosurvival cytokines, early mitochondrial injury and subsequent apoptosis results from oxidant injury which is enhanced by GC. Furthermore, GC-enhanced oxidant production is associated with prolonged c-Jun NH2-terminal kinase (JNK) activation and the loss of both mitochondrial Mn superoxide dismutase (SOD) and antiapoptotic X-linked inhibitor of apoptosis protein (XIAP). Importantly, inhibition of GC-induced apoptosis by GM-CSF and an inhibitor of JNK demonstrate the necessity of this proapoptotic mitogen-activated protein kinase in the mitochondrial injury and apoptosis that follows GC treatment.

Materials and Methods

**Eosinophil isolation and culture**

Eosinophils were isolated from mildly allergic donors and cultured according to Hoontrakoon et al. (2). To summarize, cells were isolated by a negative immunomagnetic procedure and transferred to 5-ml polypropylene tubes in 1-ml aliquots (0.5 million/ml), and cultured in Iscove’s medium (Life Technologies, Grand Island, NY) with 20% FCS (HyClone Laboratories, Logan UT). Eosinophils were then treated with various concentrations of dexamethasone (10−7–10−6 M; Calbiochem, San Diego, CA), GM-CSF (100 pg/ml; R&D Systems, Minneapolis, MN), anti-Fas IgM (Upstate Biotechnology, Lake Placid, NY), DPI, a flavoprotein inhibitor (5 μM; Ref. 28), or JNK inhibitor SP600125, (10−4 M; both from Calbiochem, San Diego, CA). Mn(III) tetrakis (5, 10, 15, 20-benzoic acid)porphyrin (MnTBAP; 200 μM) was prepared in the laboratory of Dr. B. J. Day (29). Where reagents were dissolved in DMSO or ethanol, final concentrations were kept ≤0.1%, and controls with and without the vehicle alone showed no differences. For anaerobic chamber incubations, medium was placed in 1.2-ml microtiter tubes in the chamber (Billups-Rothenberg, Del Mar, CA), the chamber was flushed with N2 and the medium was allowed to deoxygenate at 37°C for 3–4 h before addition of cells. The chamber was opened briefly for the addition or harvest of eosinophils at indicated times and refueled with N2 after each opening and returned to a 37°C incubator. Recombinant human GM-CSF (100 pg/ml; R&D Systems) was added at the start of incubation where indicated. This concentration was chosen because it consistently antagonized the effects of dexamethasone at 10−8 M, the highest dose that can be achieved in vivo during systemic steroid administration.

**Assessment of eosinophil viability and apoptosis**

Eosinophil viability was assessed at various time points by four independent methods: 1) trypan blue exclusion, 2) nuclear and cytoplasmic condensation by light microscopy following Kimura staining, 3) flow cytometric analysis of hypodiploid DNA based on propidium iodide staining of permeabilized cells, and 4) FITC-annexin V (Caltag Laboratories, Burlingame, CA)/propidium iodide staining for apoptosis (30). We have previously shown that all of these methods are highly correlated. Although there is donor-to-donor variation in apoptotic rate, the time course of each feature is consistent for any given donor: annexin V staining and nuclear morphology show the earliest evidence of apoptosis beginning at ~24 h, followed by DNA fragmentation at 32–48 h, and loss of trypan blue exclusion at 40–48 h (2).

**Mitochondrial membrane potential**

After culture, eosinophils were incubated with 10 mM JC-1 (Molecular Probes, Eugene, OR) at room temperature for 20 min, centrifuged, resuspended in cold PBS, and analyzed by flow cytometry for dye dihydroethidium fluorescence (22).

**MnSOD staining**

After culture, eosinophils were spun down, fixed in 100 μl PBS with 4% paraformaldehyde, 0.1% saponin, and 1% BSA for 30 min, then spun down...
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Statistical analysis

Statistical analysis was conducted by ANOVA using the JMP statistical program (SAS Institute, Cary, NC). The Tukey-Kramer and Dunnett’s parametrical tests were used for single and multiple comparisons, respectively.

Results

Eosinophil apoptosis and dose-dependent enhancement by GC

Though donor-to-donor variability was evident, eosinophils obtained from mildly allergic donors and cultured in the absence of prosurvival cytokines became nonviable (trypan blue-positive) over 96 h as has been seen in other studies (12, 13, 32); mean viability was 78% (±5% SD), 40% (±7% SD), and 20% (±4% SD) at 24, 48, and 72 h, respectively. Of note, this loss of viability is very similar to that documented for eosinophils obtained from normal donors (9, 26). Cell death was further enhanced by dexamethasone in a dose-dependent fashion starting at concentrations of 10−8 M, and this was not apparent until after the 24-h time point, and was near maximal at 48 h (Fig. 1). That cell death was due to apoptosis was demonstrated by the appearance of hypodiploid DNA (Fig. 1, inset) confirming previous studies (12, 13, 32). Additionally, apoptotic death was confirmed by characteristic morphologic features on light microscopy (cytoplasmic and nuclear condensation), and the externalization of phosphatidylserine as detected by FITC-annexin V in the flow cytometer (data not shown) (2).

FIGURE 2. Mitochondria injury during eosinophil apoptosis. A. Loss of mitochondria membrane potential with and without dexamethasone as shown by increased JC-1 green fluorescence at 16 h compared with 0 h in a representative histogram. B. The percentage of cells without enhanced JC-1 green fluorescence (cells with intact, uninjured mitochondria, + SD) was quantified and was found to be significantly less for cultures treated with dexamethasone compared with control at 16 h ($\alpha = 3; \ast, p < 0.05$). C. Dexamethasone addition promotes Bax activation and translocation to the mitochondria at 16 h. Eosinophils were stained with Mitotracker Red to identify healthy mitochondria, and then with the 6A7 Bax Ab which recognizes the activated form of Bax (green), and with Hoechst for nuclei (blue). Bar is 10 μm.
Apoptosis is preceded by mitochondrial damage

Injurious changes to mitochondria during the process of apoptosis have been reported for many cell types including eosinophils (10). Eosinophils were treated with and without dexamethasone for 16 h at which point they were incubated with JC-1. JC-1 green fluorescence increased concurrently with mitochondrial injury and decreased membrane potential, and preceded morphological changes of apoptosis. JC-1 green fluorescence increased in dexamethasone-treated cells when compared with control eosinophils, and this occurred in a dose-dependent manner (Fig. 2A). Healthy cells with low green fluorescence were quantified and expressed in Fig. 2B as cells with intact mitochondria (normal membrane potential). Dexamethasone treatment elicited a 30% decrease in the number of cells with intact viable mitochondria at 16 h before apoptosis could be demonstrated by either phosphatidylserine externalization or DNA analysis.

Activation/translocation of Bax was determined after 16 h in culture. Eosinophils were incubated with Mitotracker Red to identify mitochondria, fixed, and stained with the Bax Ab 6A7, which recognizes the activated epitope of Bax. Although control eosinophils began to show some 6A7 staining, eosinophils treated with dexamethasone displayed an obvious enhancement in 6A7 staining.

Antioxidant conditions inhibit eosinophil mitochondria injury and apoptosis

Although oxidant-induced injury has been noted in many cell types undergoing apoptosis, including other granulocytes (33), there is precedence both for ROS opening the mitochondrial permeability transition pore (34), and the opening of the permeability transition pore with loss of membrane potential being the source of ROS (21). Thus, to determine whether oxidant injury could be the cause or result of mitochondrial damage, eosinophils were incubated with and without dexamethasone under hypoxic conditions, or in the presence or absence of MnTBAP (a permeant catalytic antioxidant with SOD activity) (35) or the flavoprotein inhibitor, DPI (28), for 16 h and loss of mitochondrial membrane potential was analyzed by increased JC-1 green fluorescence. Cells incubated in hypoxic conditions showed a significant protection against mitochondrial injury compared with cells incubated in room air (Fig. 3A). This protection was observed in control cells undergoing spontaneous apoptosis as well as those stimulated with dexamethasone. Similar prevention of mitochondrial injury was seen when eosinophils were treated with MnTBAP (Fig. 3B), and DPI (with the later having an effect only in the presence of GC; see Discussion) (Fig. 3C). Inhibitors of nitric oxide synthase did not alter mitochondrial injury or apoptosis (data not shown).

Hypoxia or inhibition of oxidants significantly slowed both baseline apoptosis and its enhancement by GC (Fig. 3D). Eosinophils were stimulated with and without dexamethasone for 48 h under hypoxic or normoxic conditions, with DPI or MnTBAP and apoptosis assessed as trypan blue positivity (shown), DNA degradation, and nuclear morphology. Cultures incubated in decreased O_2_ or with DPI or MnTBAP, displayed significantly reduced control and GC-induced apoptosis. Anti-Fas IgM was used as a control proapoptotic stimulus to ensure that the cells could be stimulated to undergo apoptosis under hypoxic conditions; no significant difference was seen in the induction of apoptosis in hypoxic or normoxic conditions with this stimulus (data not shown).

Oxidant injury is accompanied by loss of MnSOD

Oxidant injury may be the result of increased oxidant production and/or decreased removal by antioxidant defense mechanisms. With mitochondrial injury occurring early in the apoptotic process, loss of mitochondrial MnSOD was suggested as a possible target. Additionally, GC has been shown to decrease levels of MnSOD (36, 37). To pursue this question, eosinophils were incubated for 24 h with and without dexamethasone, and then paraformaldehyde fixed, permeabilized, stained for MnSOD, and levels determined by flow cytometry. Over time, eosinophils demonstrated a significant decrease in MnSOD levels (without affecting actin levels, data not shown) compared with freshly isolated cells, indicating depletion of the naturally protective mitochondrial antioxidant protein (Fig. 4A). Furthermore, dexamethasone treatment resulted in

FIGURE 3. Antioxidant conditions inhibit mitochondrial injury and apoptosis. A, Hypoxic vs normoxic conditions protect eosinophil mitochondria with or without dexamethasone treatment at 24 h as analyzed by JC-1 green fluorescence. Histograms are representative of four experiments. B and C, Pretreatment (20 min) with the SOD mimetic, MnTBAP, or flavoprotein inhibitor, DPI, protect mitochondria from GC-enhanced damage at 16 h as determined by JC-1 green fluorescence. MnTBAP also protects mitochondria under control conditions. Histograms are representative of three experiments. D, Enhanced viability of eosinophils is demonstrated by trypan blue exclusion at 48 h. * Significant differences between treatment group and respective normoxic control (n = 3–6).
additional depletion of MnSOD. Flow cytometry data was confirmed with Western blots for MnSOD; conversely, Western blotting for the cytosolic antioxidant protein, CuZnSOD, showed no differences with time in culture or with dexamethasone treatment (data not shown).

To demonstrate production of oxidants, eosinophils were incubated with and without dexamethasone and then analyzed by flow cytometry for dihydroethidium red fluorescence. Eosinophils treated with $10^{-8} - 10^{-6}$ M dexamethasone for 24 h showed enhanced dihydroethidium red fluorescence compared with control eosinophils (Fig. 4A) paralleling observations in GC-induced treated T cells (19, 21). Of note, comparisons at earlier time points did not show consistently detectable oxidant production with dexamethasone treatment over controls (data not shown), and unfortunately, the methodology did not allow for comparisons of cells analyzed at different points in time in culture (see Discussion).

**GC-induced oxidants lead to JNK activation and JNK-mediated apoptosis**

Oxidants can lead to JNK activation, which has been associated with induction of apoptosis in other cells (38, 39), and dexamethasone has been shown previously to activate JNK in eosinophils (13). To determine whether JNK activation was due to the elicited oxidants, eosinophils were treated with and without dexamethasone in the presence or absence of DPI. DPI was chosen for these experiments as DPI appeared to be more specific in inhibiting the effects of GC (had less effect on control cultures) than MnTBAP (see Fig. 3 and Discussion). Cells were stimulated for 40 min at which point they were lysed and lysates were run on a 10% SDS-PAGE gel. Blots were probed with an anti-phospho-JNK Ab. Although JNK did not appear to be activated in control cells at this time point, dexamethasone significantly stimulated JNK phosphorylation compared with untreated cells in a dose-dependent manner (Fig. 5A). As predicted, this activation of JNK was inhibited with the antioxidant DPI. These findings suggest that ROS production begins within minutes following dexamethasone, although it is undetectable using dihydroethidium (above).

Because JNK activation has been linked to mitochondrial damage in other cells (Refs. 38 and 39, and see Discussion), we asked whether JNK activation was required for mitochondrial injury observed with and without GC treatment. Eosinophils were stimulated with and without dexamethasone in the presence of various doses of the JNK inhibitor, SP600125, and mitochondrial membrane potential was assessed at 16 h by JC-1 fluorescence. Notably, this anthrapyrazolone has been shown to inhibit JNKs 1, 2, and 3 with little or no effect on the kinase activity of ERK-1 and P38 (40). Although the JNK inhibitor appeared to have minimal effect on control cells, GC-induced mitochondrial damage (loss of...
membrane potential) was completely blocked in samples treated with the JNK inhibitor (Fig. 5B). Importantly, Bax activation/translocation enhanced by GC treatment was also attenuated with the JNK inhibitor, SP600125, 20 min before addition of dexamethasone. JC-1 green fluorescence histograms are representative of three independent experiments. C, Inhibition of JNK activity prevents dexamethasone-induced activation of Bax. Cells were treated with dexamethasone in the presence or absence of the JNK inhibitor, 100 μM for 16 h. Samples were then stained for Bax activation with Ab 6A7 (green) and with Hoechst for nuclei (blue), and analyzed by fluorescent microscopy. Bar is 10 μm. D, Dexamethasone-enhanced apoptosis is prevented with the JNK inhibitor in a dose-dependent manner. Cells were pretreated with the JNK inhibitor (20 min) before dexamethasone, 10⁻⁶ M, was added for 24–30 h. Apoptosis was determined by annexin V binding. Bars represent the percent of nonapoptotic eosinophils (annexin V/propidium iodide-negative) + SD (n = 3). *, Significantly less apoptosis was seen when the JNK inhibitor (50 or 100 μM) was added to the dexamethasone-treated cultures (p < 0.05).

Recent reports in other cell types have suggested that JNK activation is inhibited by XIAP (41, 42), and that XIAP levels are reduced by dexamethasone treatment (43). We hypothesized that JNK activation might be prolonged by GCs and that GC-induced loss of XIAP might contribute to this prolonged activation. In support of this hypothesis, JNK activation was confirmed at 16 h in dexamethasone-treated, but not control, eosinophils (Fig. 6A). Furthermore, dexamethasone treatment for 16 h was found to clearly reduce XIAP levels compared with control, and this occurred in a dose-dependent manner (Fig. 6B). Therefore, it appears that GC treatment not only activates JNK within minutes by an oxidant-dependent mechanism, but also reduces the levels of the JNK inhibitor, XIAP, possibly contributing to prolonged JNK activation.

FIGURE 5. Dexamethasone-enhanced apoptosis is dependent on JNK activation. A, Dexamethasone treatment (40 min) stimulates JNK phosphorylation in cultured eosinophils. Pretreatment with DPI (20 min) before dexamethasone inhibits JNK phosphorylation. Immunoblot is representative of four independent experiments. B, Inhibition of JNK activity prevents GC-induced mitochondria damage at 16 h: eosinophils were cultured with 100 μM of the JNK inhibitor, SP600125, 20 min before addition of dexamethasone. JC-1 green fluorescence histograms are representative of three independent experiments. C, Inhibition of JNK activity prevents dexamethasone-induced activation of Bax. Cells were treated with dexamethasone in the presence or absence of the JNK inhibitor, 100 μM for 16 h. Samples were then stained for Bax activation with Ab 6A7 (green) and with Hoechst for nuclei (blue), and analyzed by fluorescent microscopy. Bar is 10 μm. D, Dexamethasone-enhanced apoptosis is prevented with the JNK inhibitor in a dose-dependent manner. Cells were pretreated with the JNK inhibitor (20 min) before dexamethasone, 10⁻⁶ M, was added for 24–30 h. Apoptosis was determined by annexin V binding. Bars represent the percent of nonapoptotic eosinophils (annexin V/propidium iodide-negative) + SD (n = 3). *, Significantly less apoptosis was seen when the JNK inhibitor (50 or 100 μM) was added to the dexamethasone-treated cultures (p < 0.05).
GM-CSF addition inhibits GC-induced mitochondrial damage and subsequent apoptosis

Prosurvival HGSFs, and particularly GM-CSF, have been implicated in inhibiting eosinophil apoptosis in vitro and in vivo (2, 4, 5, 12, 44). It has been shown that IL-5 (in the absence of GC) inhibits Bax activation/translocation, mitochondrial damage, cytochrome c release, caspase activation, and subsequent apoptosis (10, 12). As has been previously reported (32), incubating eosinophils with GC in the presence of GM-CSF blocked GC-induced apoptosis (Fig. 7A), and as hypothesized, GM-CSF also prevented loss of mitochondrial membrane potential as determined by JC-1 green fluorescence (Fig. 7, B and C). Additionally, Bax activation/translocation initiated by GC treatment were almost completely prevented by GM-CSF as seen by a decrease in 6A7 staining (Fig. 7D). Mitochondrial membrane integrity was also restored, as seen by increased Mitotracker Red fluorescence in GM-CSF-stimulated cells.

GM-CSF treatment does not inhibit GC-induced oxidant production or loss of MnSOD

The potent inhibition of GC-induced apoptosis by GM-CSF led to the possibility that this prosurvival cytokine prevents GC-induced oxidant production. To pursue this question, eosinophils were loaded with the oxidant-sensitive dye dihydroethidium and then stimulated with dexamethasone in the presence or absence of GM-CSF (Fig. 8A). Although dexamethasone treatment significantly enhanced dihydroethidium fluorescence compared with control, GM-CSF treatment did not prevent this enhancement, indicating that the mechanism(s) by which GM-CSF inhibits apoptosis is(are) downstream of oxidant production.

To determine whether inhibition of GC-induced apoptosis by GM-CSF was due to enhanced mitochondrial oxidant defense, MnSOD expression was determined. Although, GM-CSF stimulation alone appeared to have little effect on the level of MnSOD over time in culture, the combination of GM-CSF with dexamethasone did not preserve MnSOD levels (Fig. 8B). Actin staining showed no difference in protein levels between samples (data not shown). Thus preservation of GC-treated eosinophils by GM-CSF could not be attributed either to changes in oxidant production or antioxidant MnSOD levels.

GM-CSF inhibits prolonged GC-induced JNK activation and preserves XIAP expression

As shown above, dexamethasone stimulated JNK activation as early as 40 min, and prolonged activation out to 16 h. Due to the association of GC-induced JNK activation with eosinophil apoptosis (Fig. 5), JNK became a prime candidate for prosurvival cytokine targeting. As shown above, dexamethasone treatment resulted in early JNK activation (Fig. 9). Of note, GM-CSF also activated JNK at 40 min, an effect that is likely attributable to activation of the NADPH oxidase (45) and oxidant production implicated in the GM-CSF prosurvival signaling pathways that ultimately serve to protect mitochondria (46, 47) (Fig. 9). Although dexamethasone-stimulated JNK phosphorylation at 40 min was not affected by GM-CSF treatment, GM-CSF did, however, inhibit the prolonged JNK activation stimulated by dexamethasone at 16 h (Fig. 9, A and B).

We hypothesized that GM-CSF’s inhibition of prolonged JNK activation induced by GC might be due to preservation of XIAP. In support of this hypothesis, GM-CSF preserved XIAP levels in the presence of dexamethasone (Fig. 9C). Thus, the data are consistent with inhibition of prolonged JNK activity as an important target of HSGF prosurvival signaling which may be mediated, at least in part, through the maintenance of the JNK inhibitor, XIAP.

Discussion

Oxidant production and early mitochondrial injury

Apoptosis in eosinophils is envisioned as a three-step process: a premitochondrial phase involving oxidant-mediated effects; a mitochondrial phase during which mitochondrial membrane function is lost and cytochrome c release, caspase activation, and subsequent apoptosis (19, 21) and superantigen-induced mitochondrial injury (19, 21, 26). These data strongly suggest that production of ROS is an essential early event of the premitochondrial phase of eosinophil apoptosis (upstream of caspase activation), is responsible for mitochondria damage, and is enhanced by GC (Figs. 2–4). As such, our findings in eosinophils parallel GC-induced oxidant production (19, 21) and superantigen-induced mitochondrial injury (likewise inhibited by MnTBAP), both shown to be early events in T cell apoptosis (22).

The source(s) of oxidant production is as yet undetermined. That DPI treatment inhibited caspase activation in eosinophils (with or without GC treatment), and that caspase activation is enhanced by GC in eosinophils after 24–48 h of culture (12, 13, 26). These data strongly suggest that production of ROS is an essential early event of the premitochondrial phase of eosinophil apoptosis (upstream of caspase activation), is responsible for mitochondria damage, and is enhanced by GC (Figs. 2–4). As such, our findings in eosinophils parallel GC-induced oxidant production (19, 21) and superantigen-induced mitochondrial injury (likewise inhibited by MnTBAP), both shown to be early events in T cell apoptosis (22).

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have occurred. GC treatment may block MnSOD transcription because both AP-1 and NF-κB sites have been found in the MnSOD gene promoter (51, 52), and a requirement for NF-κB in transcription has been demonstrated (52, 53). Importantly, a role for MnSOD in preventing mitochondrial injury is suggested in MnSOD−/− knockout mice where the deletion is embryonically lethal (54), and in heterozygous mutant mice which have increased release of cytochrome c in the cytosol compared with wild-type mice (MnSOD+/−) following ischemic injury (55) or aging (56). Of note, while MnSOD loss may well contribute to oxidant injury and cytochrome c release in eosinophils (especially following GC treatment), MnSOD did not appear essential for survival when eosinophils were also treated with GM-CSF which maintained mitochondrial integrity independent of the oxidant production during GC-treatment. These findings are in keeping with those of Peachman et al. (9) who demonstrated that the energy needs of eosinophils are met largely by glycolysis rather than respiration.

**GC-induced JNK activation enhances mitochondrial injury**

JNK, a member of the mitogen-activated protein family of serine-threonine kinases activated by stress, (and particularly within minutes following oxidant stress), is thought to contribute to apoptosis in many cell types (38, 39, 57). Of note, we did not find evidence for JNK activation in eosinophil apoptosis in the absence of GC treatment. Conversely, and similar to the findings of Zhang et al. (13), GC activated JNK within minutes, and we have shown that this was dependent on early ROS production (Fig. 5). Additionally, we found that JNK phosphorylation was prolonged to 16 h with GC treatment (Fig. 6A). Although Zhang et al. (13) showed that JNK1/2 antisense phosphorothioate oligodeoxynucleotides suppressed dexamethasone-induced JNK activity at 15 min, these investigators did not find inhibition of apoptosis. However, suppression of JNK at later time points was not assessed. In addition, these experiments required 12 h for oligodeoxynucleotide incorporation and the effect

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**FIGURE 7.** GM-CSF treatment protects eosinophils from GC-induced apoptosis. A, Eosinophils were treated with dexamethasone in the presence or absence of 100 pg/ml GM-CSF and cell death was assessed by trypan blue staining. Graph represents the percent of viable cells ± SD (n = 6). Cells treated with GM-CSF, with or without dexamethasone, showed significantly less apoptosis than control cells or cells treated with dexamethasone alone at 48–72 h by ANOVA (*, p < 0.05). B, Mitochondria injury induced by dexamethasone at 16 h is inhibited by GM-CSF as determined by JC-1 green fluorescence in a representative histogram. C, The percentage of cells (+ SD) with intact mitochondria (low JC-1 green fluorescence) is significantly lower for cells treated with dexamethasone alone than those treated with GM-CSF with or without dexamethasone; (n = 5; *, p < 0.05). D, GM-CSF inhibits dexamethasone-induced activation of Bax. Eosinophils were stimulated as in B, stained with Mitotracker Red for mitochondria, fixed, and stained with the activated Bax Ab 6A7 (green) and Hoechst (blue) for nuclei. Cells were analyzed by fluorescent microscopy. Bar is 10 μm.
of dexamethasone was assessed only after an additional 12 h. Our data show that a newly available inhibitor of JNK protected mitochondria and inhibited GC-induced apoptosis (Fig. 5). Furthermore, inhibition of apoptosis was still evident even when the inhibitor was added 4 h after GC addition (data not shown) suggesting that it is late or prolonged JNK activation that is critical for GC enhancement of apoptosis. Of note, our preliminary experiments in thy- 

mocytes from mitogen/extracellular-regulated kinase kinase 1/ 

H11002 mice, demonstrate that loss of this upstream activator of JNK also inhibits GC-induced apoptosis by 50% (unpublished data).

Mounting evidence points to the mitochondrion as a down- 

stream target of activated JNK in the induction of apoptosis. Targeted disruptions of functional jnk genes protect fibroblasts from UV-induced loss of mitochondrial membrane potential, cyto- 

chrome c release, and apoptosis (58). Our findings show that in- 

hibition of JNK activation prevents the activation of Bax and mitochondrial injury (Fig. 5). Although regulation of Bax translo- 

cation from cytosol to mitochondria is poorly understood, it may involve loss of heterodimerization with antiapoptotic Bcl-2 family members allowing Bax to self-associate as a mitochondrial membrane pore-forming multimer (59). In other cells, activated JNK has been shown to associate with, and phosphorylate, the antiapoptotic proteins Bcl-2 and Bcl-xL and alter their effects. Phosphory- 

lation of the latter has been shown to lead to mitochondrial injury and apoptosis; while overexpression of Bcl-xL, with mutated sites blocking phosphorylation by JNK, inhibited mitochondrial injury and apoptotic death (38, 39). Thus it is suggested that JNK activa- 

tion may alter the functional balance and activities of these anti- and proapoptotic Bcl-2 family members in eosinophils.

In addition to reducing MnSOD levels, GC treatment also re- 

sulted in loss of the antiapoptotic protein, XIAP, a member of the antiapoptotic IAP family. The IAPs were originally described as inhibitors of caspases (60), but recently, one member of this protein family, XIAP, has also been shown to inhibit JNK by the sequestration of elements required for its activation (42). In other systems, GC and oxidants have been shown to enhance ubiquitin-proteosome activity (61, 62), and to specifically induce the self- 

ubiquitination of XIAP and related c-IAP1 in thymocytes (43). Our data in eosinophils demonstrates that XIAP is lost with GC treat- 

ment and its loss is associated with prolonged JNK phosphoryla- 

tion (Fig. 6). Although definitive studies are not possible in the short-lived, terminally differentiated eosinophil, overexpression of IAPs or expression of ubiquitination-resistant forms in other cells have been shown to inhibit GC-induced apoptosis (43).

GM-CSF maintains XIAP, inhibits both prolonged JNK activation and Bax activation/translocation, and protects mitochondria

To date, the mechanism(s) by which the HSGFs inhibit eosinophil apoptosis is not fully understood. Notably, definitive studies in these nondividing, relatively scarce cells are difficult. Levels of Bcl-2, Bcl-xL, and Bax (the latter two being the predominant anti-
and proapoptotic family members, respectively, in blood eosinophils; Refs. 63 and 64) did not show consistent change in our hands (data not shown) confirming the results of others (10, 12). Rather than changes in the levels of expression of these proteins, it was the activation and translocation of Bax to mitochondria that was associated with loss of mitochondrial membrane potential and cytochrome c release in eosinophils cultured without HSGFs (10). Dawson et al. (10) found that both Bax translocation and associated mitochondrial injury were independent of caspase activation and were inhibitable by IL-5. Here, GM-CSF similarly inhibited Bax activation/translocation, subsequent mitochondrial injury, and apoptosis, both in control cultures and in cultures where mitochondrial injury was significantly enhanced by GC (Fig. 7). Although GM-CSF did not inhibit either oxidant production (Fig. 8) or early JNK activation, it did inhibit prolonged JNK activation (Fig. 9) which we have shown is required for enhanced Bax activation/translocation during GC treatment (Fig. 5C). Our finding that GM-CSF also leads to maintenance of XIAP (Fig. 9), and reports that XIAP inhibits JNK (41, 42), suggest a possible mechanism for these observations. Whether GM-CSF maintains XIAP by stimulating synthesis or inhibiting degradation, or both, will require further study.

Without question, GCs are the most effective form of treatment for asthma and other atopic diseases, though mounting data suggest that a subgroup of patients are resistant to GC treatment. Studies suggest that GCRβ, an alternative splice variant of the GCR which does not bind GC, antagonizes the transactivation activity of the classic GCR and may be implicated in GC resistance (65, 66). The recent finding of GCRβ in eosinophils from sinus biopsies of GC-resistant sinusitis patients (67) parallels the finding of GCRβ in T cells from GC-resistant asthmatic patients (66), and GC-insensitive neutrophils (68). Whether GC-induced oxidant production, JNK activation, and mitochondrial injury will differ in eosinophils from GC-resistant subjects is the focus of ongoing research. A number of studies indicate that GC resistance results in a lack of suppression of Th2 cytokine production at the level of T cells and possibly other cells as well (66, 69, 70). Thus, we would hypothesize that in such individuals, ongoing production of GM-CSF and IL-5 would contribute to resistance to GC-induced suppression of eosinophil differentiation, recruitment, priming, and ultimately, apoptosis.

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References


