Role of the CD95/CD95 Ligand System in Glucocorticoid-Induced Monocyte Apoptosis

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Glucocorticoids (GC) act as potent anti-inflammatory and immunosuppressive agents on a variety of immune cells. However, the exact mechanisms of their action are still unknown. Recently, we demonstrated that GC induce apoptosis in human peripheral blood monocytes. In the present study, we examined the signaling pathway in GC-induced apoptosis. Monocyte apoptosis was demonstrated by annexin V staining, DNA laddering, and electron microscopy. Apoptosis required the activation of caspases, as different caspase inhibitors prevented GC-induced cell death. In addition, the proteolytic activation of caspase-8 and caspase-3 was observed. In additional experiments, we determined the role of the death receptor CD95 in GC-induced apoptosis. CD95 and CD95 ligand (CD95L) were up-regulated in a dose- and time-dependent manner on the cell membrane and also released after treatment with GC. Costimulation with the GC receptor antagonist mifepristone diminished monocyte apoptosis as well as CD95/CD95L expression and subsequent caspase-8 and caspase-3 activation. In contrast, the caspase inhibitor N-acetyl-Asp-Glu-Val-Asp-aldehyde suppressed caspase-3 activation and apoptosis, but did not down-regulate caspase-8 activation and expression of CD95 and CD95L. Importantly, GC-induced monocyte apoptosis was strongly abolished by a neutralizing CD95L mAb. Therefore, our data suggest that GC-induced monocyte apoptosis is at least partially mediated by an autocrine or paracrine pathway involving the CD95/CD95L system. The Journal of Immunology, 2001, 166: 1344–1351.

Glucocorticoids (GC) are known as potent immunosuppressive and anti-inflammatory agents with the potential to inhibit the expression of several cytokines involved in inflammatory responses (1–3). Their effect is mediated by the cytosolic GC receptor which, upon ligand binding, translocates into the nucleus and regulates gene expression (4, 5). As monocytes and macrophages carry many GC receptors, they are highly sensitive to GC treatment (6). Down-regulation of a variety of monocyte proinflammatory mediators including TNF-α (3), IL-1β (2, 7), IL-6 (1, 8), or IL-8 (9) by GC has been reported. Recently, we could demonstrate that GC induce monocyte apoptosis (10), which may explain monocytopenia that is observed during GC therapy (11, 12). It is conceivable that at least some of the anti-inflammatory properties described for GC may be attributed to the induction of monocyte apoptosis.

Under serum-free culture conditions monocytes will rapidly undergo apoptosis (13). Stimulation with proinflammatory mediators such as TNF-α, IL-1β, or LPS prevents monocyte apoptosis (14, 15). We have recently demonstrated that continuous treatment with IL-1β almost completely abolished GC-induced monocyte apoptosis (10). However, it remains still unclear whether the observed down-regulation of proinflammatory cytokines follows GC-induced monocyte apoptosis or is an initial proapoptotic signal.

Monocytes express detectable levels of death receptor CD95 and CD95 ligand (CD95L) on their membranes (13). Recently, it has been demonstrated that endogenous expression of CD95 and its ligand CD95L plays a role in spontaneous apoptosis, since blocking CD95 ligation prevented apoptosis of monocytes in culture (13). It was further shown that activated monocytes release CD95L (16, 17) suggesting that the CD95 pathway is involved in autocrine and paracrine monocyte apoptosis. The apoptotic pathway mediated by CD95 is well-defined (18, 19). Triggering of the receptor by its ligand or agonistic Abs induces a death-inducing signaling complex that consists of the adapter protein Fas-associated death domain protein and caspase-8 (20, 21). Caspase-8 is the most proximal element in the caspase cascade. Further downstream in the death pathway, caspase-8 triggers the proteolytic activation of other caspases and the cleavage of various cellular substrates, thereby mediating the apoptotic response (21–23).

As induction of apoptosis in monocytes seems to be an essential anti-inflammatory process, knowledge of its underlying mechanisms is of great importance. Especially in patients with immunological diseases, who are resistant to GC therapy or those who suffer from GC adverse effects, a better insight into the mechanisms of apoptosis may be of relevance for the development of therapeutic strategies.

In this study we investigated the signaling pathway of GC-induced monocyte apoptosis. We demonstrate that GC-induced apoptosis is associated with an enhanced expression of membrane-bound CD95 and CD95L as well as an increased release of both molecules from the cell surface. Treatment of monocytes with GC was followed by the proteolytic activation of caspase-8 and caspase-3. GC-induced apoptosis was prevented by caspase inhibition as well as by neutralizing CD95L Abs. Our data suggest that GC trigger monocyte apoptosis in an autocrine or paracrine manner involving a CD95-dependent signaling pathway.

Acknowledgments

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Abbreviations used in this paper: GC, glucocorticoids; CD95L, CD95 ligand; DEVD-CHO, N-acetyl-Asp-Glu-Val-Asp-aldehyde; PARP, poly(ADP-ribose)polymerase; PL, propidium iodide; zVAD-fmk, benzoyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone; Ac-IETD-AFC, N-acetyl-Ile-Glu-Thr-Asp-7-amino-4-trifluoromethyl coumarin.

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Materials and Methods

Abs and reagents

PE-conjugated mouse anti-human Leu M3 mAb (anti-CD14, clone P9, IgG2b) and control mAbs of appropriate isotypes were obtained from Becton Dickinson (Palo Alto, CA). FITC-labeled annexin V was purchased from Bender Medsystems (Vienna, Austria). The broad caspase inhibitor benzylxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk) was obtained from Promega (Mannheim, Germany), and the caspase-3 inhibitor N-acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO) from Calbiochem (Bad Soden, Germany). Anti-human CD95 mAb was purchased from R&D Systems (Wiesbaden, Germany), anti-human CD95-L was provided from PharMingen BD (Heidelberg, Germany) (65320C mouse IgG1 NOK-1) (24, 25) and anti-poly(ADP-ribose)polymerase (PARP) rabbit serum from Roche Molecular Research (Mannheim, Germany). Anti-caspase-8 mAb and a neutralizing anti-CD95L mAb (clone 5G51, mouse IgG1) were provided by BioCheck (Münster, Germany). A colorimetric caspase assay was obtained from Promega. A Western blot analysis system was received from Amersham Pharmacia Biotech (Freiburg, Germany). All other reagents were obtained from Sigma (St. Louis, MO).

Isolation and culture of human monocytes

Human monocytes were isolated from 40 ml EDTA-treated blood, drawn from healthy volunteers or from fresh leukocyte buffy coats. We used a modification of the recently described isotonic density gradient centrifugation method with Ficoll and Percoll (26). Briefly, mononuclear cells were collected from the interphase after Ficoll separation and washed twice in PBS. Subsequently, cells were separated into lymphocytes and monocytes on an isotonic Percoll density gradient (1.129 g/ml). From the two interphases the upper interphase containing monocytes was collected and washed three times with PBS. The monocyte suspension was adjusted to 1 × 10^6 cells/ml and plated on 24-well plates (Greiner, Solingen, Germany). Anti-human CD95 mAb was purchased from R&D Systems (Wiesbaden, Germany), anti-human CD95-L was provided from PharMingen BD (Heidelberg, Germany) (65320C mouse IgG1 NOK-1) (24, 25) and anti-poly(ADP-ribose)polymerase (PARP) rabbit serum from Roche Molecular Research (Mannheim, Germany). Anti-caspase-8 mAb and a neutralizing anti-CD95L mAb (clone 5G51, mouse IgG1) were provided by BioCheck (Münster, Germany). A colorimetric caspase assay was obtained from Promega. A Western blot analysis system was received from Amersham Pharmacia Biotech (Freiburg, Germany). All other reagents were obtained from Sigma (St. Louis, MO).

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SAP and reagents

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Detection of annexin V staining and CD14 expression

Monocytes, prepared and treated as described above, were double-labeled with PE-conjugated Leu M3 mAb (anti-CD14) and annexin V-FITC in PBS for 1 h at room temperature in the dark. PE-conjugated murine IgG mAbs of unrelated specificity were always used as control. After staining, the cells were washed twice in PBS and measured by flow cytometry.
Determination of apoptosis by DNA electrophoresis

DNA extraction and electrophoresis were performed as described previously (10). In brief, $1 \times 10^7$ monocytes were first lysed in a hypotonic buffer (10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.2% Triton X-100). After centrifugation ($14,000 \times g$, 30 min), supernatants containing cleaved chromatin were treated with 50 mg/ml RNase and 100 mg/ml proteinase K, and DNA was extracted by phenol/chloroform/isoamylalcohol. After precipitation with ethanol at $-20^\circ C$ and drying and heating of the samples, equal amounts of DNA were loaded on a 1.8% agarose gel and separated by electrophoresis for 2 h at 80 V.

Transmission electron microscopy

Morphological alterations indicative of apoptosis were evaluated by transmission electron microscopy. Cells were washed off the culture plates, centrifuged, fixed in 1% glutaraldehyde/0.1 M Na cacodylate (pH 7.4), and postfixed in 1% OsO4/0.15 M Na cacodylate (pH 7.4). Samples were dehydrated in ascending ethanol series and embedded in epoxy resin (Epon 812). Ultrathin sections were mounted on 150 mesh Formvar-coated copper grids and poststained with aqueous saturated uranyl acetate and 2% lead citrate before being examined on a Philips CM 10 electron microscope (Philips Electronic Instruments, Mahway, NJ) at an accelerating voltage of 60 kV.

Immunoblotting

The proteolytic activation of caspase-8 and cleavage of PARP were detected by immunoblotting. Following 24 h of culture, monocytes were lysed in 100 µl Laemmli sample buffer containing 2% SDS, 125 mM Tris-HCl (pH 6.8), 20% glycerol, and 1% 2-ME. After centrifugation, 50 µg total protein of each sample was then incubated with the colorimetric caspase-3 substrate Ac-DEVD-pNA. The release of the yellow chromophore pNA (p-nitroanilide) was measured in a spectrophotometer at 405 nm. For caspase-8 assay, monocytes were lysed in the same manner. Total protein (200 µg) of each sample was then incubated with the colorimetric caspase-8 substrate Ac-IETD-AFC. Ac-IETD-AFC is a synthetic tetrapeptide substrate that is cleaved by active human caspase-8. This substrate is cleaved between D and AFC, releasing the fluorogenic AFC, which is detected by spectrophotometer at 490 nm.

Analysis of CD95 and CD95L secretion

Cell culture supernatants were collected after 24 h. CD95 and CD95L were determined in an equal volume of each sample. For immunoblot analysis a standard procedure was used as described above.

Statistical analysis

Results are given as means ± SD. For statistical analysis Student’s unpaired t test was used. Statistical significance was considered if $p < 0.05$. All experiments were performed at least ten times with different blood donors, unless otherwise indicated.
Results

Treatment with GC induces monocyte apoptosis

Monocytes were cultured for 48 h and treated with different doses of dexamethasone (10^{-10}, 10^{-8}, and 10^{-6} M). Apoptosis could be demonstrated by three independent methods including annexin V staining, DNA laddering, and transmission electron microscopy (Fig. 1). GC treatment of the monocytes increased the number of annexin V-positive cells in a dose-dependent fashion. After incubation with 10^{-6} M dexamethasone, the percentage of apoptotic cells increased from 15.7 ± 4.1% in the medium control to 53.7 ± 11.4% in GC-treated monocytes (Fig. 1A). Agarose gel electrophoresis showed that marked DNA laddering typical for apoptosis was already detectable in monocytes treated with a dose of 10^{-6} M dexamethasone (Fig. 1B).

Morphological alterations of dexamethasone-treated monocytes were determined by electron microscopy. Fig. 1C shows a healthy control monocyte with a normal relation of the nucleus and cytoplasm as well as intact organelles. In contrast, cells treated with different concentrations of dexamethasone revealed typical features of apoptosis including cytoplasmatic and chromatin condensation and a marked vacuolization of the cytoplasm (Fig. 1, D–F).

To differentiate between apoptotic and necrotic cells, we performed double staining with annexin V-FITC and the DNA dye PI. As shown in Fig. 2, monocytes treated with different doses of dexamethasone revealed increased annexin V staining, but no membrane damage. After 48 h of cell culture, we found 10.0% single-positive monocytes in the medium control (Fig. 2A). GC treatment increased the number of annexin V-positive and PI-negative cells to 17.7%, 19.9% and 42.9% at concentrations of 10^{-10} M, 10^{-8} M and 10^{-6} M dexamethasone, respectively (Fig. 2, B–D). In contrast, 94.5% of the cells permeabilized with saponin became double-positive for PI and annexin V (Fig. 2E). Therefore, the data show that dexamethasone induces apoptotic, but not necrotic cell death in human monocytes.

Dexamethasone-induced apoptosis is GC receptor and caspase dependent

To investigate the specificity of the observed effects, we performed experiments with the GC receptor antagonist mifepristone (27). Fig. 3 shows that simultaneous treatment with GC and mifepristone blocked monocyte apoptosis, as the number of annexin V-positive cells decreased from 47.3% to 20.7%. We further investigated the involvement of caspases in dexamethasone-induced apoptosis. Pretreatment of cells with DEVD-CHO, a peptide inhibitor more selective for caspase-3, significantly attenuated apoptosis, and the number of annexin V-positive cells was reduced almost 2-fold (Fig. 3). Using a colorimetric substrate assay we found that treatment of monocytes with different concentrations of dexamethasone resulted in the conversion of the 116-kDa full-length form of PARP into the characteristic p85 fragment (Fig. 4B). However, in control monocytes, the p85 fragment was only weakly detectable.

We further studied the activation of caspase-8/Fas-associated death domain protein-like IL-1β-converting enzyme, which is the most proximal regulatory caspase during death receptor-mediated cell death. Caspase-8 is synthesized as an inactive precursor of 54 kDa and, following formation of 43- and 41-kDa intermediate cleavage products, processed to a p18 and p10 heterodimer. Treatment of monocytes with dexamethasone resulted in the conversion of pro-caspase-8 to the 43- and 41-kDa intermediate fragments as well as to the p18 and p10 subunits (Fig. 5A). The results indicate that activation of caspase-8 is involved in GC-induced monocyte apoptosis. Upon simultaneous treatment with dexamethasone and mifepristone, the p43 and p41 lanes as well as the p18 and p10 bands were only weakly expressed. Caspase-8 activation could also be demonstrated by a colorimetric assay that was increased in monocytes after treatment with GC. Addition of mifepristone as well as zVAD-fmk almost completely abolished the effect of GC on monocytes (Fig. 5B).

Increase of CD95 and CD95L expression after GC treatment

Dexamethasone-induced caspase-8 activation suggesting the possible involvement of CD95 and CD95L in GC-induced apoptosis. Monocytes and macrophages are known to express both CD95 and CD95L on their membrane surface (13). To investigate a potential involvement of the CD95 system in GC-induced apoptosis, we determined CD95 and CD95L expression on monocyte surface by FACS analysis. Treatment of monocytes with dexamethasone led to increase of the surface expression of both CD95 and CD95L as
demonstrated by changes in the mean fluorescence (Fig. 6). Cotreatment with the GC receptor antagonist mifepristone inhibited the inducible and also the constitutive expression of CD95 and CD95L. In contrast, simultaneous treatment of monocytes with dexamethasone and the caspase inhibitor DEVD-CHO diminished monocyte apoptosis (see Fig. 2), but did not block the up-regulation of membrane-bound CD95 and CD95L (Fig. 6).

Since both molecules may be also released from the cell surface, we further analyzed the expression of CD95 and CD95L in culture supernatants by immunoblot analysis. CD95 is a transmembrane receptor type I with 45 kDa whereas the CD95L is a transmembrane receptor type II with 40 kDa. Only the extracellular part of the receptor will be shed into the supernatant; therefore, the expected molecular mass of the soluble part of the receptors is between 20 and 35 kDa (31–33). CD95 and CD95L-specific protein bands were strongly enhanced in samples treated with GC, indicating a marked release of CD95 and CD95L (Fig. 7). Cotreatment with mifepristone inhibited the release of CD95 and CD95L into supernatants of GC-treated monocytes.

Treatment with anti-CD95L Abs reduces GC-induced apoptosis

To examine the functional relevance of the CD95 system in GC-induced monocyte apoptosis, we treated monocytes simul-aneously with various doses of dexamethasone together with either agonistic anti-CD95 mAb or neutralizing anti-CD95L mAb. After 48 h, monocytes were stained with annexin V-FITC and measured by FACS analysis. As expected, anti-CD95-mAb significantly enhanced monocyte apoptosis in the medium control as well as in GC-treated cultures (Fig. 8). In contrast, neutralizing CD95L by anti-CD95L mAb decreased GC-induced monocyte apoptosis; incubation with 10⁻⁶ M dexamethasone resulted in 38.4 ± 6.1% annexin V-positive cells, while cotreatment with anti-CD95L reduced the amount of apoptotic cells to 24.3 ± 4.9%. These results suggest that CD95/CD95L interaction is functionally involved in GC-induced cell death of monocytes.

Discussion

In the present study, we demonstrate that GC induce monocyte apoptosis by a CD95-dependent signaling pathway. We were able to show an up-regulation of membrane-bound CD95 receptor and ligand as well as the release of both molecules after GC treatment. This process is followed by the activation of caspase-8 and cleavage of PARP by activated caspase-3. Neutralization of CD95L diminished GC-induced monocyte apoptosis suggesting that the inducible expression and subsequent interaction of CD95 and CD95L play an important role in GC-induced apoptosis.
GC exert immunosuppressive and anti-inflammatory effects on different cell types including monocytes and macrophages. Some of the anti-inflammatory actions induced in monocytes are caused by the down-regulation of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6 (1–3, 7, 8), as well as by repression of NF-κB activation (34, 35). Induction of apoptosis in various cell populations, such as lymphocytes, as a potential immunomodulating mechanism of GC action has been described earlier (36–38). Recently, we have demonstrated that GC are capable of inducing apoptosis also in human monocytes (10). However, the signaling pathway responsible for GC-induced monocyte apoptosis remained unknown so far.

As spontaneous monocyte apoptosis was reported to be mediated at least in part by a mechanism requiring CD95 and CD95L (39–40), we determined the potential involvement of those molecules in GC-induced apoptosis. Monocytes express CD95 and CD95L on their membrane surfaces (13). Under serum-free conditions, monocytes increase membrane-bound CD95 and CD95L, thereby inducing autocrine and paracrine cell death (13). This process can be abolished by blocking CD95L. It has also been shown that monocytes contain high levels of intracellular CD95L, which can be released or expressed on the surface membrane after cellular activation (16, 17, 41). Therefore, it was postulated that CD95 ligation plays an important role in monocyte apoptosis. In our study we found a significant enhancement of CD95 and CD95L expression on monocyte surface membranes as well as an increased release into cell culture supernatants after treatment with GC. This mechanism seemed to be directly induced by GC, as blocking of the GC receptor with mifepristone abolished up-regulation of CD95 and CD95L expression. By contrast, caspase inhibition with DEVD-CHO did not exert any effect on the surface expression of both molecules. The presented data reveal representative results for eight independent experiments that showed similar results.

![FIGURE 6. GC enhance CD95 and CD95L expression on monocyte membranes. Monocytes were cultured for 24 h and treated with 10−8 M dexamethasone. As determined by flow cytometry, the mean fluorescence of CD95 (A) and CD95L (B) was enhanced by GC. Mifepristone significantly inhibited GC-induced up-regulation of CD95 and CD95L. In contrast, caspase-inhibition with DEVD-CHO did not exert any effect on the surface expression of both molecules. The presented data reveal representative results for eight independent experiments that showed similar results.](http://www.jimmunol.org/)

**A**

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Mean fluorescence CD95

Mean fluorescence CD95-L

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The mechanisms by which GC induce apoptosis are presumably controlled by cell type-specific processes. In thymocytes and T cell hybridomas, for instance, GC induce apoptosis by mechanisms which are independent of CD95. Paradoxically, in these cells dexamethasone protects against activation-induced cell death by suppressing the expression of CD95 and CD95L (47). An inhibition of the inductive expression of these molecules has also been observed for ligands of related nuclear receptors, such as retinoids, which, similarly to GC, protect against activation-induced cell death of T-cells, but induce apoptosis in several other cell types (47). It is known that CD95L and other cytokines of the TNF family are transcriptionally regulated in a cell type- and context-specific manner (48). Future experiments have to elucidate which transcriptional activators or control elements regulate CD95 and CD95L expression in response to GC in monocytes. It is interesting to note that the effects of GC are strikingly opposite in different liver cell types (49). While dexamethasone diminished LPS-induced stimulation of CD95L expression in nonparenchymal liver cells, it markedly stimulated CD95L expression in parenchymal cells.

Apoptosis induced in monocytes by immunosuppressive steroids may be an important mechanism in the treatment of chronic inflammatory diseases. Systemic monocytopenia, which is observed following steroid treatment, may be a consequence of an increased rate of monocyte apoptosis (11, 12). While our data clearly demonstrate that GC induce apoptosis by a CD95-dependent pathway, our results do not exclude that additional mechanisms may exist. GC are known as potent monocyte deactivators that down-regulate proinflammatory cytokine expression. As a result, the inducible expression of these molecules has also been observed for ligands of related nuclear receptors, such as retinoids, which, similarly to GC, protect against activation-induced cell death of T-cells, but induce apoptosis in several other cell types (47). It is known that CD95L and other cytokines of the TNF family are transcriptionally regulated in a cell type- and context-specific manner (48). Future experiments have to elucidate which transcriptional activators or control elements regulate CD95 and CD95L expression in response to GC in monocytes. It is interesting to note that the effects of GC are strikingly opposite in different liver cell types (49). While dexamethasone diminished LPS-induced stimulation of CD95L expression in nonparenchymal liver cells, it markedly stimulated CD95L expression in parenchymal cells.

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