Regulation of Granulocyte-Macrophage Colony-Stimulating Factor and E-Selectin Expression in Endothelial Cells by Cyclosporin A and the T-Cell Transcription Factor NFAT

By Gillian W. Cockerill, Andrew G. Bert, Gregory R. Ryan, Jennifer R. Gamble, Mathew A. Vadas, and Peter N. Cockerill

Nuclear factor of activated T cells (NFAT) was originally described as a T-cell-specific transcription factor that supported the activation of cytokine gene expression and mediated the immunoregulatory effects of cyclosporin A (CsA). As we observed that activated endothelial cells also expressed NFAT, we tested the antiinflammatory properties of CsA in endothelial cells. Significantly, CsA completely suppressed the induction of NFAT in endothelial cells and inhibited the activity of granulocyte-macrophage colony-stimulating factor (GM-CSF) gene regulatory elements that use NFAT by 60%. CsA similarly mediated a reduction of up to 65% in GM-CSF mRNA and protein expression in activated endothelial cells. CsA also suppressed E-selectin, but not vascular cell adhesion molecule-1 (VCAM-1) expression in endothelial cells, even though the E-selectin promoter is activated by NF-κB rather than NFAT. Hence, induction of cell surface expression of this leukocyte adhesion molecule by tumor necrosis factor (TNF-α) was reduced by 40% in the presence of CsA, and this was reflected by a 29% decrease in neutrophil adhesion. The effects of CsA on endothelial cells were also detected at the chromatin structure level, as DNase1 hypersensitive sites within both the GM-CSF enhancer and the E-selectin promoter were suppressed by CsA. This represents the first report of NFAT in endothelial cells and suggests mechanisms by which CsA could function as an antiinflammatory agent.

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sively on endothelial cells that interacts with receptors on a subset of leukocytes that include neutrophils, eosinophils, monocytes, and a subset of memory T cells. E-selectin is involved in the earliest stages of leukocyte recruitment as it mediates the initial tethering and rolling of leukocytes across the endothelium.

Acute and chronic inflammatory conditions are typically maintained by the activation of both cytokines and adhesion molecules that function in concert to orchestrate the interactions between numerous participating cell types. Elevated expression of E-selectin, for example, is associated with inflammatory conditions such as asthma, rheumatoid arthritis, and psoriasis. While CsA is more commonly used as an immunosuppressive agent to prevent organ transplant rejection, it has also been found to be effective in the treatment of a variety of chronic inflammatory and autoimmune disorders such as psoriasis, asthma, ulcerative colitis, and rheumatoid arthritis. Some of the benefits of these treatments can be attributed to actions against T cells, there may be additional direct effects on either the activation or trafficking of cell types such as neutrophils, eosinophils, and monocytes.

To gain a better understanding of the involvement of the endothelium in inflammation, we have investigated the regulation of the GM-CSF and E-selectin genes in endothelial cells. For this purpose we have used passaged human umbilical vein endothelial cells (HUVEs) and the recently derived endothelial cell line C1 lSTH. We initially cloned C1 lSTH cells from a spontaneous transformant that arose in a culture of HUVEs and showed that they have a repertoire of adhesion molecules and TNF-α receptors similar to that found in normal HUVEs.

In this study we found that, as in T cells, the GM-CSF enhancer was required for efficient activation of the GM-CSF promoter in endothelial cells. Interestingly, these effects appeared to be mediated in part by the transcription factor NFAT. Furthermore, Nfat and GM-CSF induction in endothelial cells was suppressed by CsA. The induction of E-selectin in endothelial cells and acquired ability to support neutrophil adhesion were also inhibited by CsA, suggesting a wider role for this drug in combating inflammation. In the course of these studies we established that the endothelial cell line C1 lSTH is a valuable model system for studying inflammatory events in the endothelium.

MATERIALS AND METHODS

Reagents. The phorbol ester phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma (St Louis, MO), the calcium ionophore A23187 (I) was purchased from Boehringer (Mannheim, Germany), forskolin was purchased from Calbiochem (San Diego, CA), cyclosporin A (CsA) was a gift from Sandoz (Basel, Switzerland), DNase I was purchased from Worthington (Freehold, NJ), and TNF-α and interferon-γ (IFN-γ) were provided by Genetech (San Francisco, CA). PMA was prepared as a 1-mg/mL stock in dimethyl sulfoxide (DMSO). A23187 as a 20-mmol/L stock in DMSO, forskolin as a 10-mmol/L stock in DMSO, and CsA as a 1-mmol/L stock in ethanol.

Oligonucleotides. Oligonucleotide duplexes used as probes and competitors had the following sequences, with complementary single stranded regions at each end shown in lower case for the upper strand only: GM430, gatcTACACATCTTCTCATTGGAAGATGA; IL-2 NFAT, gatcCGAAAGGGAAAAACTGTTTCTACAGAAG; X, TGCCTAAGTGCTCCGGGTGCACTGCAGAAGCTTC; Igκ NF-κB, AAGCAGAGGACCTTTCCGAGCCATCT; E-selectin NF-κB, aattCGTTTTTGGATGCCATGAGAAG; X, TCACACATCTTTCTCATGGAGATcGTTTTTGGATGCCATGAGAAGAGATgCTACACATCTTCTCATTGGAAGATG.

Plasmids. The luciferase reporter gene plasmid pGMLuc had a 655-bp fragment of the human GM-CSF promoter (−627 to +28) upstream of the luciferase gene. The plasmid pGMELuc has a 716-bp BglII fragment of the human GM-CSF enhancer placed upstream of the GM-CSF promoter in the BglII site of pGMLuc.

Cell culture. C1 lSTH cells and HUVEs were cultured as previously described.

Antibodies. The antibody R59 was raised against recombinant truncated NFATp prepared from pNFATpX12. The antibody 67.5 was raised against peptide 72 of purified mouse NFATp12. These antibodies were a gift from A. Rao12 and cross-react between human and mouse NFATp. The cc-1 antibody was a gift from R. Stun (Centre for Molecular Biology and Biotechnology, Brisbane, Australia).

Gel electrophoretic mobility shift assays. Gel shift assays of nuclear extracts were essentially as previously described10 and used 0.2 ng of 32P-labeled probe, 5 μg of nuclear protein, and 2 μg of poly(dI-dC) in a 15-μL vol. Nuclear extracts were prepared as previously described11 from unstimulated cells and cells stimulated for 2 to 3 hours with 20 ng/mL PMA and 2 μmol/L A23187 (I) in the presence and absence of 0.1 μmol/L CsA. CsA was added 10 minutes before the addition of PMA and A23187. Assays of recombinant proteins differed in that they used 200 ng poly(dI-dC), 4 μmol/L dithiothreitol and 0.3 mg/mL bovine serum albumin together with 0.5 ng NFATp, 10 ng NF-κB p50, or 0.07 μL NF-κB p65. The NFATp was a 293-amino acid truncated derivative of NFATp containing the DNA-binding domain and was prepared from the plasmid pQE-31#1 (a gift from A. Rao), which is derived from pNFATpX12 by expression in Escherichia coli and chromatography on Qiaogen NT + NTA agarose according to manufacturer’s instructions. NF-κB p50 was purchased from Promega (Madison, WI), while the NF-κB p65 used here was a gift of purified recombinant truncated protein (amino acids 1-313) supplied by S. Gerondakis (Wajar and Eliza Hall Institute, Melbourne, Australia).

GM-CSF enzyme-linked immunosorbent assay. GM-CSF protein concentrations in cell culture supernatants were determined according to manufacturer’s instructions using a human GM-CSF enzyme-linked immunosorbent assay (ELISA) kit supplied by R & D Systems (Minneapolis, MN). Supernatants were harvested from cultures containing 2 × 106 cells seeded in 24-well trays with 400 μL medium. Cultures were initially incubated for 24 hours and then activated for 16 hours as indicated.

GM-CSF mRNA assays. GM-CSF mRNA levels were assayed as previously described14 after stimulation of cells for 8 hours with 20 ng/mL PMA and 2 μmol/L A23187 (I) in the presence and absence of 0.1 μmol/L CsA. Briefly, RNA was prepared by the guanidine lysis method and assayed by RNase protection assay. Each hybridization reaction contained probes for both the GM-CSF and the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes, and the levels of GM-CSF expression were expressed relative to GAPDh.

DNase I hypersensitive site analysis. DNase I hypersensitive sites (DH) sites in C1 lSTH cells, HUVEs, Jurkat cells, and peripheral blood T lymphocytes were assayed as described16 after stimulating the cells for 4 to 6 hours with 20 ng/mL PMA and 2 μmol/L A23187 (I) or 100 U/mL TNF-α in the presence and absence of 0.1 μmol/L CsA. Filters were first hybridized with the GM-CSF probe,
then stripped by washing in 50% formamide at 65°C, before rehy-
bridizing with the E-selectin probe. The E-selectin probe was a 1.7-
kB {\textit{BglII}}/EcoRI fragment normally located 1.3 to 3 kb downstream of the human E-selectin gene transcription start site, and was a gift from T. Collins (Brigham and Women's Hospital, Boston, MA). T lymphocytes were freshly purified from peripheral blood by Ficoll centrifugation to isolate mononuclear cells, elutriation to remove monocytes, and nylon wool chromatography to remove B cells.

Transfection assays. Luciferase reporter gene activities were de-
termined as previously described by transfecting 20 μg of each plas-
mid construct into 1 × 10⁴ C11STH cells by the diethylaminoethyl (DEAE) dextran procedure and measuring luciferase expression after stimulation with 2 μmol/L A23187 (I) and 20 ng/ml PMA for 9 hours in the presence and absence of 0.1 μmol/L CsA.

Neutrophil adhesion assays. Neutrophil adhesion assays were performed as previously described. Briefly, confluent monolayers of C11STH cells were incubated with 100 U/ml TNF-α in the presence or absence of 1 μmol/L CsA. After 4 hours’ activation, ¹²⁵I-labeled neutrophils (9 × 10⁶ cpm) were added and incubated at room temperature (approximately 22°C) for 15 minutes. After several washes with medium, neutrophil adherence was estimated by measuring bound ¹²⁵I.

E-selectin and VCAM-1 expression. Cell surface expression of E-selectin and VCAM-1 on C11STH cells was determined by flow cytomeric (FACS) analysis using specific antibodies for E-selectin and VCAM-1, as previously described. Before analysis, cells were stimulated for 4 hours with different combinations 100 U/ml IFN-γ, 100 U/ml TNF-α, 20 ng/ml PMA, 2 μmol/L A23187 (I), and 10 μmol/L forskolin.

RESULTS

Activated endothelial cells express NFAT. CsA is a po-
tent immunosuppressive drug known to function in T cells by inhibiting induction of the transcription factor NFAT. Although there have been no previous reports of NFAT in endothelial cells, CsA is used as an antiinflammatory agent, and some of its actions could be mediated by suppression of factors in the endothelium. Therefore, we have examined whether NFAT can be induced in endothelial cells by pathways known to activate NFAT in T cells. To this end, we stimulated C11STH cells and passaged HUVEs with a combina-
tion of the phorbol ester PMA and the calcium ionophore A23187. These signals mimic the phospholipase C-driven activation of protein kinase C and increase in cytosolic Ca²⁺ and induce the AP-1 and NFATp/c components of NFAT, respectively.

We performed gel electrophoretic mobility shift assays of nuclear extracts prepared from activated HUVEs and C11STH cells using the GM-CSF enhancer GM430 se-
quence as a probe for NFATp/c binding (left hand panel in Fig 1) and the human IL-2 gene distal NFAT site as a probe for NFAT (right hand panel in Fig 1). The IL-2 site served as the prototypic NFAT site most commonly used to assay for the presence of NFAT in T cells as it cooperatively binds AP-1 and NFATp/c to form NFAT complexes. The GM430 site is a high-affinity NFATp site that is able to associate strongly with the NFATp/c component of NFAT independently of AP-1.⁹

Significantly, nuclear extracts from HUVEs and C11STH cells both formed inducible NFATp/c-like complexes with the GM430 element and NFAT-like complexes with the IL-2 NFAT site that comigrated with Jurkat T-cell complexes in gel mobility shift assays (Fig 1). As in T cells, CsA completely inhibited the induction of these endothelial cell NFAT and NFATp/c-like complexes (Fig 1). Furthermore, complex formation with both probes was completely inhibited when either the IL-2 or GM430 NFAT elements were included as DNA competitors, but only partially inhibited when an unrelated competitor (X) was used.

NFATp appeared to be the major protein contributing to NFATp/c-like complexes with the GM430 probe in C11STH and HUVE extracts as antibodies raised against either recombinant NFATp or to an NFATp peptide specifically inhibited formation of or super-shifted NFATp-like complexes as effi-
ciently as they did with Jurkat cell extracts (Fig 2). Addition of a 5- to 20-fold greater amount of oct-1 antiseraum had little effect on NFATp complex formation with the endothelial cell nuclear extracts. As the 67.1 NFATp antibody was raised against a peptide not conserved in NFATc, there appeared to be little or no NFATc present in either the endothelial cell or the Jurkat cell extracts employed in this study.

NFATp induction in endothelial cells may require a Ca²⁺ signal not provided by proinflammatory cytokines, as TNF-α did not induce NFATp (data not shown). In contrast, TNF-α was a more efficient inducer of NFATp in endothelial cells than PMA and A23187 (data not shown).

GM-CSF expression is regulated by CsA in endothelial cells.

If NFATp regulates gene expression in endothelial cells then GM-CSF gene activation via Ca²⁺ pathways in endothelial cells should also be CsA-sensitive. To explore this possibility, we examined GM-CSF synthesis in four independent lines of passaged HUVEs. As in T cells, the level of GM-CSF secretion by HUVEs was increased 100-fold by the combined actions of the phorbol ester PMA and the calcium ionophore A23187 (PMA/I, Fig 3). For each of the four lines of activated HUVEs, there was a decrease in GM-CSF expression in the presence of 0.1 μmol/L CsA, with an average reduction of 50%.

As we anticipated that CsA would inhibit GM-CSF at the transcriptional level, we also performed RNase protection assays of GM-CSF mRNA in HUVEs and C11STH cells. Just as we observed for GM-CSF protein synthesis, GM-CSF mRNA was strongly induced in both HUVEs and C11STH cells by PMA and A23187 (Fig 4). GM-CSF mRNA induction required calcium ionophore as well PMA, as sixfold less GM-CSF mRNA was detected in the presence of PMA alone. In the presence of 0.1 μmol/L CsA the induc-
tion of GM-CSF mRNA was reduced by 65% in HUVEs and 56% in C11STH cells (Fig 4). This inhibition appeared to be specific, as we observed no effect of CsA on IL-8 expression in endothelial cells (data not shown). CsA also appeared to have little effect on induction of GM-CSF expression by pathways that do not use Ca²⁺. Hence, CsA had no significant influence on induction of GM-CSF mRNA or protein expression in endothelial cells activated with TNF-α (data not shown).

The GM-CSF enhancer exists as a DNase1 hypersensitive site in endothelial cells.

In T cells, the GM-CSF enhancer exists as an inducible CsA-sensitive DNase1 hypersensitive (DH) site that spans at least two NFAT sites. To identify
a role for this enhancer in the GM-CSF locus in endothelial cells, we examined DH sites upstream of the GM-CSF gene. These analyses were performed both in C11STH cells (Fig 5A) and in passaged HUVEs (Fig 5B).

We located two DH sites that coincided with the proximal promoter and the previously defined enhancer 3 kb upstream of the gene. The DH site located within the promoter appeared as a constitutive site in both C11STH cells (Fig 5A) and HUVEs (Fig 5B). Although not detected in Jurkat T cells, this site also appeared as a DH site in activated T lymphocytes (Fig 5B). The DH site within the enhancer appeared as a weak site before activation and was strongly induced in the presence of the coactivators PMA and A23187 in both C11STH cells and HUVEs. The DH site existed as a broad region 2.8 to 3.0 kb upstream of the GM-CSF gene, thus resembling the DH site induced in T cells. Significantly, induction of this DH site was reduced by about 70% in the presence of 0.1 μmol/L CsA, which had no effect on the promoter DH site (Fig 5A). We also examined endothelial cells activated by TNF-α, but observed that this cytokine was not a significant inducer of any DH sites within the GM-CSF locus (data not shown).

The GM-CSF enhancer contributes to GM-CSF activation in endothelial cells. To determine whether the upstream enhancer was required for efficient GM-CSF promoter activation in endothelial cells, the enhancer was coupled to the GM-CSF promoter in a luciferase reporter gene plasmid. On transfection into C11STH cells and stimulation with the coactivators PMA and A23187, the enhancer supported a fivefold greater induction of luciferase gene expression than was obtained with a luciferase gene driven by the GM-CSF promoter alone (Fig 6). Furthermore, this induction was suppressed by about 60% in the presence of CsA.

CsA inhibits neutrophil adhesion to activated endothelial cells. Having found that CsA could suppress cytokine gene expression, we next examined whether CsA could influence neutrophil adhesion to activated endothelial cells. Neutrophils become involved at the earliest stages during the progression of inflammation and are also one of the cell types activated by GM-CSF.

In the presence of TNF-α, we observed an approximately sixfold increase in the adherence of neutrophils to a confluent monolayer of C11STH cells (Table 1). In the presence of CsA, we observed a 29% decrease (P = .0006) in the TNF-α–induced neutrophil adherence.

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**Fig 1.** Gel electrophoretic mobility shift assays of NFATp and NFAT in endothelial cell nuclear extracts. Nuclear extracts were prepared from passaged HUVEs, C11STH cells, and Jurkat cells that were either unstimulated (nil) or stimulated for 2 to 3 hours with 20 ng/mL PMA and 2 μmol/L A23187 in the presence (PMA/I/CsA) or absence (PMA/I) of 0.1 μmol/L CsA. Bands migrating below the NFATp and NFAT complexes appear to represent nonspecific constitutive factors. Where indicated, assays of stimulated C11STH cell nuclear extract binding to the GM430 NFATp and IL-2 NFAT probes also include 25 ng duplex oligonucleotides corresponding to the GM430 NFATp site, the IL-2 NFAT site, or an unrelated sequence (X) as competitor. Each assay used 5 μg protein, and the asterisk indicates one lane where a shorter autoradiographic exposure was included to assist identification of the NFATp complex in the Jurkat extract.

**Fig 2.** Gel electrophoretic mobility shift assays of nuclear extracts binding to the GM430 NFATp probe in the presence of NFATp antibodies. Assays used 1 μg stimulated Jurkat cell nuclear extract or 5 μg stimulated C11STH or HUVE nuclear extract. Extracts were assayed either with no added anti-sera (nil) or after preincubation for 20 minutes on ice with 0.2 μL of preimmune serum, 0.2 μL R59 antisera raised against recombinant NFATp, 0.05 μL 67.1 antisera raised against an NFATp peptide, or 1 μL of an antisera raised against oct-1. The 67.1-antisera produced super-shifted complexes characteristic of specific NFATp binding and does not cross-react with NFATc.
Fig 3. Suppression of GM-CSF protein synthesis in HUVEs by CsA. Levels of GM-CSF secretion by cultured HUVEs were measured by ELISA. Four independent lines of HUVEs were either not stimulated (NIL) or stimulated for 16 hours with 20 ng/mL PMA and 2 μmol/L A23187 in the presence (PIC) or absence (PI) of 0.1 μmol/L CsA. Shown above are the mean expression levels obtained with the four lines of HUVEs together with error bars indicating the SEM. There was a statistically significant mean decrease of 320 pg/mL GM-CSF (SEM = 132, \( P < .01 \)) in the four lines of HUVEs in the presence of CsA.

E-selectin expression is suppressed by CsA in endothelial cells. To determine which leukocyte adhesion molecules on endothelial cells might be suppressed by CsA, we examined the regulation of E-selectin and VCAM-1 expression in C11STH cells. E-selectin can interact with receptors on neutrophils, eosinophils, monocytes, and subsets of T cells and is an important regulator of both acute and chronic inflammation.23,24 VCAM-1 plays a major role in the adherence of monocytes and lymphocytes to endothelial cells.23

We first showed that E-selectin was induced in C11STH cells in the expected fashion by either TNF-α or a combination of PMA and A23187 (PMAII) (Fig 7). IFN-γ alone was unable to induce E-selectin expression, but greatly enhanced its activation by TNF-α. We also observed that the cAMP inducer forskolin was a potent inhibitor of TNF-α-inducible E-selectin expression in C11STH cells, as previously observed by others.23 The second DH site, located 3.5 kb upstream, appeared as a constitutive DH site. Significantly, the inducible but not the constitutive site, was suppressed by CsA (Fig 8A), suggesting that CsA-sensitive transcriptional activation ele-

Fig 4. Suppression of GM-CSF mRNA expression in endothelial cells by CsA. RNA was isolated from HUVEs and C11STH cells that were either unstimulated (Nil), stimulated for 8 hours with 20 ng/mL PMA (PMA), or stimulated for 8 hours with 20 ng/mL PMA and 2 μmol/L A23187 in the presence (PMA/I/CsA) or absence (PMA/I) of 0.1 μmol/L CsA. GM-CSF mRNA levels were assayed by RNase protection assay, using GAPDH as an internal control.
ments existed in the proximal promoter. Both DH sites appeared to be endothelial cell specific as they did not appear in activated Jurkat cells or T cells (Fig 8B) or in a wide range of other cell types examined (data not shown).

CsA may suppress NF-κB activity in the E-selectin promoter. We examined the sequence of the E-selectin promoter seeking transcription factor binding sites that might be involved in DH site formation and be suppressed by CsA. Previous studies of E-selectin gene regulation have implicated an array of three NF-κB sites in the promoter as being crucial for activation of E-selectin expression, and these sites may be the most likely targets for the actions of CsA in this locus. Indeed, there is some evidence that NF-κB p50 and c-rel expression is inhibitable by either CsA or FK506, which might account for the 40% inhibition of E-selectin expression that occurs in the presence of CsA. Nevertheless, there is also evidence obtained with T cells that NFAT-like factors can in some cases associate with NF-κB-like regulatory regions.

To determine whether NFAT or NF-κB proteins are more likely to account for the CSA-sensitivity of E-selectin expression, we examined binding of recombinant NFATp, NF-κB p50, and NF-κB p65 to the best characterized NF-κB site in the E-selectin promoter (PDI), and to three control NFATp and NF-κB binding sites (Fig 9). This particular E-selectin NF-κB site was the most proximal of the three NF-κB sites in the promoter and the one found to be the most essential for E-selectin promoter activity. As this element encompasses the sequence AGAGGAAA, it is also the E-selectin NF-κB site that most resembles binding sites for NFATp.

As anticipated from earlier studies of T-cell nuclear extracts, both the IL-2 NFAT site and the Igκ NF-κB site had the capacity to associate with all three proteins. This confirmed the view that NF-κB and NFATp have the poten-

Table 1. Suppression of Neutrophil Adhesion to C11STH Cells by CsA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Adhesion (cpm × 10^4)</th>
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<tbody>
<tr>
<td>Nil</td>
<td>117 ± 7 (n = 7)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>795 ± 33 (n = 14)</td>
</tr>
<tr>
<td>TNF-α/CsA</td>
<td>565 ± 49 (n = 9)</td>
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Results are presented as mean ± SEM. The 29% decrease in neutrophil adhesion in the presence of CsA was statistically significant as a Student’s t-test gave P = .0006.
CsA suppresses induction of E-selectin expression in endothelial cells. E-selectin expression was induced in C115TH cells as indicated above in the presence of increasing concentrations of CsA, which was added 10 minutes before stimulation of the cells. See Materials and Methods for activation conditions.

Fig 7. CsA suppresses induction of E-selectin expression in endothelial cells. E-selectin expression was induced in C115TH cells as indicated above in the presence of increasing concentrations of CsA, which was added 10 minutes before stimulation of the cells. See Materials and Methods for activation conditions.

CsA (μM) 0 0.01 0.1 1
Nil IFN TNF IFN

Fig 8. Mapping of DH sites upstream of the E-selectin gene in endothelial cells from an EcoRI site 3.0 kb upstream of the transcription start site. Cells were either unstimulated (Nil) or stimulated for 4 to 6 hours with 20 ng/ml PMA and 2 μmol/L A23187 in the presence (P/I) or absence (P/I) of 0.1 μmol/L CsA or with 100 U/ml TNF-α. (A) shows DH sites in C115TH cells. Note that these sites are not detected in the absence of DNase1 digestion (control DNA). The inducible DH site in the proximal region of the promoter appeared as a 3.0-kb fragment, whereas an upstream constitutive DH site appeared as a 6.5-kb fragment. The membranes used in (A) and (B) are the same as those in Fig 3.

Fig 9. Gel electrophoretic mobility shift assays of recombinant NFATp and NF-κB. The indicated probes were incubated with 0.5 ng NFATp, 10 ng NF-κB p50, or 0.07 μL NF-κB p65. The probes corresponded to the IL-2 promoter distal NFAT site, the κ Ig gene NF-κB site, the E-selectin promoter proximal NF-κB site, and the GM430 high-affinity NFATp site from the GM-CSF enhancer.
many early inflammatory events, and CsA, which is in widespread use as an immunosuppressive and antiinflammatory drug. Two important classes of molecules that are activated during the early stages of inflammation are the cytokines and adhesion molecules that direct the activation and trafficking of leukocytes that enter inflammatory lesions. We have concentrated on GM-CSF and E-selectin as potential targets for antiinflammatory actions of CsA in endothelial cells, and these are two molecules that might function in concert on neutrophils and eosinophils.

Surprisingly, we discovered that immunoregulatory mechanisms initially thought to be restricted to lymphoid cells also operated in endothelial cells. Hence, the T-cell transcription factor NFAT was induced in endothelial cells by pathways that in T cells are triggered by T-cell receptor and phospholipase C activation. CsA, an immunosuppressant previously thought to function primarily by inhibiting induction of NFAT and cytokine gene expression in T cells, also inhibited activation of NFAT in endothelial cells. The endothelial cell NFAT complexes were found to contain the protein NFATp, which has recently been reported to be expressed in both hematopoietic and nonhematopoietic cells, rather than NFATc, which is thought to be lymphoid-specific. Although there are few previous reports suggesting functions for NFAT outside of the T-cell lineage, a recent study has implicated NFAT as a factor required for TNF-α gene expression in B cells. Other studies have demonstrated that ligation of surface immunoglobulin results in CsA-sensitive induction of NFAT and activation of NFAT-responsive reporter genes in B cells.

GM-CSF is a proinflammatory cytokine having the potential to activate neutrophils, monocytes, and eosinophils. Of all the cytokines that can be expressed by endothelial cells, GM-CSF is perhaps the only one strongly inhibited by CsA in T cells. Therefore, we have explored the potential of CsA to act as an antiinflammatory agent working to suppress activation of myeloid cells by GM-CSF. We observed that CsA suppressed the Ca2+-mediated activation of GM-CSF protein expression in endothelial cells by 50% and GM-CSF mRNA expression by approximately 60%.

To determine the molecular basis for the regulation of GM-CSF expression in endothelial cells, we examined the functions of regulatory elements within the GM-CSF locus. Previous studies in T cells suggested that GM-CSF gene activation is mediated to a large extent by binding of NFAT to the upstream enhancer and binding of NFAT, AP-1, and NF-κB to the promoter. In this study we found that the upstream enhancer was also required for efficient activation of the GM-CSF promoter in transfected endothelial cells, and that the activity of the promoter and enhancer was suppressed twofold in the presence of CsA. As seen previously in T cells, stimulation with PMA and A23187 led to chromatin structure changes within the enhancer that were suppressed by CsA. These observations may suggest a direct role for NFAT in the induction of the GM-CSF enhancer in endothelial cells.

In contrast to T cells, endothelial cells appeared to have only a partial dependence on NFAT for induction of GM-CSF expression. Although CsA completely suppressed induction of NFAT, it only partially suppressed the GM-CSF promoter and enhancer and GM-CSF mRNA and protein expression in endothelial cells. In T cells, CsA essentially eliminates GM-CSF gene expression. Furthermore, TNF-α was a potent inducer of NF-κB and GM-CSF expression in endothelial cells, but was a poor activator of NFAT and an inefficient inducer of the DH site in the enhancer (data not shown). Therefore, it is likely that TNF-α induces GM-CSF expression by using factors that activate the promoter in the absence of NFAT. Interestingly, the GM-CSF promoter existed as a constitutive DH site in endothelial cells (Fig 5), but not in T cells (data not shown). Consequently, the GM-CSF promoter may exist in a primed state in cultured endothelial cells and have a reduced dependence on the enhancer for its activation.

We also found some evidence suggesting that CsA can inhibit endothelial cell functions by suppressing factors other than NFAT. E-selectin expression was inhibited by about 40% in the presence of CsA by a process unlikely to directly involve NFAT. The E-selectin promoter is activated principally by an array of NF-κB sites, which represented poor binding sites for NFATp. Furthermore, the suppression of E-selectin by CsA was not limited to activation pathways involving Ca2+. CsA reduced E-selectin expression by the same extent regardless of whether TNF-α or PMA/A23187 was used to induce expression. This was in marked contrast to the GM-CSF gene, which was suppressed by CsA when activated by PMA/A23187, but not when activated by TNF-α. CsA also suppressed induction of the DH site that forms over the array of NF-κB sites in the E-selectin promoter, suggesting that CsA may regulate members of the NF-κB family in endothelial cells. This suggestion is supported by reports that CsA and FK506 can partially suppress two NF-κB family members in lymphoid cells. Indeed, the NF-κB p105/p50 gene promoter may itself be regulated by CsA, as it includes the sequence TGGACGCGATGA-CTCTA that closely resembles the NFAT consensus sequence. Alternatively, CsA may suppress E-selectin expression at the posttranscriptional level, thus accounting for the ability of CsA to suppress distinct pathways to the same extent. However, E-selectin induction via PMA was not significantly influenced by CsA, arguing against a posttranscriptional mechanism.

The evidence accumulated in this study suggests that the ability of CsA to act as an antiinflammatory drug may be partly caused by direct effects on the endothelium. Although immune and inflammatory disorders clearly involve complex cytokine networks operating within a confederacy of immune and nonimmune cell types, there is a significant role that the endothelium plays in their progression. By suppressing GM-CSF and E-selectin expression in endothelial cells, CsA could reduce the extent to which neutrophils and eosinophils are recruited at sites of inflammation. In this study, the effective dose of CsA (0.1 μmol/L) was well within the range of the plasma concentration of approximately 0.3 μmol/L CsA that is usually maintained therapeutically. Hence, this study may, in part, account for the reduction in neutrophil and eosinophil infiltration observed on treatment of disorders such as psoriasis and asthma with CsA.
ACKNOWLEDGMENT

We are indebted to A. Rao for providing NFATp anti-sera and DNA clones for NFATp, Y. Khew-Goodall for the E-selectin NF-κB probe, and T. Collins for providing E-selectin DNA clones. We thank R. Himes, F. Jenkins, and F. Shannon for the luciferase reporter gene plasmids. We thank L. Noack for technical assistance with neutrophil adhesion assays and B. Stein for assistance with statistical analyses. We thank D. Gillis, Y. Khew-Goodall, and M.F. Shannon for helpful advice and critical reading of the manuscript.

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Regulation of granulocyte-macrophage colony-stimulating factor and E-selectin expression in endothelial cells by cyclosporin A and the T-cell transcription factor NFAT

GW Cockerill, AG Bert, GR Ryan, JR Gamble, MA Vadas and PN Cockerill