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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The regulation of cytokine gene expression is central to the regulation of the immune response. The T cell has been the most intensively studied model system for investigating cytokine gene regulation, as a wide variety of cytokines are induced on activation of the T-cell receptor. The transcription factor nuclear factor of activated T cells (NFAT) plays a key role in the activation of many of these genes and is itself induced on T-cell receptor activation. The induction of cytokines such as interleukin-2 (IL-2) and granulocyte-macrophage colony-stimulating factor (GM-CSF) in T cells can be suppressed by cyclosporin A (CsA), and these effects are largely caused by the ability of CsA to inhibit activation of NFAT. Furthermore, the immunosuppressive actions of CsA have been widely attributed to its ability to suppress T-cell expression of cytokines such as IL-2, and hence T-cell activation and proliferation.

NFAT, initially thought to be a T-cell specific factor, typically consists of a complex of AP-1 family proteins and NFATp or NFATc, which are respectively induced via protein kinase C and Ca²⁺ signals originating from T-cell receptor activation. NFATp and NFATc are related proteins that are expressed in the cytoplasm of T cells and translocate to the nucleus in response to the Ca²⁺ activation of the CsA–inhibitable phosphatase calcineurin. Hence, NFATp and NFATc are major targets for the actions of CsA in T cells. The genes for NFATp and NFATc have recently been cloned and is now believed that NFATc is largely restricted to lymphoid tissues, while NFATp expression is more widespread.

NFAT contributes to the activation of the GM-CSF locus in T cells by interacting with four strong binding sites in an upstream enhancer and two weak binding sites in the GM-CSF promoter. However, GM-CSF is also expressed at sites of inflammation in many cell types other than T cells. This raises the possibility that induction of GM-CSF expression in these cell types could also require NFAT. Consequently, the GM-CSF locus may also be a target for CsA in cells such as activated endothelial cells that express GM-CSF.

The endothelial cell represents the interface between the hematopoietic compartment where immune effector cells originate and tissues where primary immune responses occur, and endothelial cells play a direct role in governing the traffic of leukocytes across the endothelium. On activation, endothelial cells produce numerous cytokines, including GM-CSF, which activate immune response effector cells such as neutrophils, monocytes, basophils, and eosinophils. Furthermore, GM-CSF may be one of the mediators of acute and chronic inflammatory conditions such as rheumatoid arthritis, asthma, and psoriasis. GM-CSF is typically induced in endothelial cells by proinflammatory cytokines such as tumor necrosis factor-α and IL-1 and its expression is regulated at both transcriptional and posttranscriptional levels. Agents that activate the protein kinase C and Ca²⁺ pathways in endothelial cells would also be expected to induce GM-CSF expression.

In addition to producing cytokines, activated endothelial cells also express the cell surface adhesion molecules E-selectin, P-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1), which mediate recruitment and transmigration of leukocytes across the endothelium. E-selectin (previously termed ELAM-1) is a cell surface glycoprotein found exclu-

From the Division of Human Immunology, Hanson Centre For Cancer Research, Institute for Medical and Veterinary Science, Adelaide, Australia.

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Address reprint requests to Peter N. Cockerill, Division of Human Immunology, Hanson Centre for Cancer Research, Institute for Medical and Veterinary Science, PO Box 14, Rundle Mall, Frome Rd, Adelaide 5000, Australia.

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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 C11STH. We initially cloned C11STH 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 C11STH 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), DNasel 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, gatcTCACACATCTTTCTCATGGAAGATGA; IL-2 NFAT, gatcCGAGGGGAAAAACGTGGTCACTAGAAG; X, TGCACTGACTGCTGCGCAGAGCTTCCGAGACCACC; Igc NF-κB, AACAGGAGGGATTTCCCGAGAGCCACCT; E-selectin NF-κB, aattCGTTTTTGGATGCCATGCATACAGAAG; X, TGCGCGTGCCGGTCCTTGAGCACCT; NF-κB p50, or 0.07 pg of poly(dI-dC), 4 mmol/L dithiothreitol and 0.3 mg/mL bovine serum albumin together with 0.5 ng NF-KB p50, or 0.07 pg TNF-α 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 mmol/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 Escherichia coli. NF-κB p50 was purchased from Promega (Madison, WI), while the NF-κB p65 used here was a gift of purified recombinant protein (amino acids 1-313) supplied by S. Gerondakis (Wajer 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. NF-κB p50 was purchased from Promega (Madison, WI), while the NF-κB p65 used here was a gift of purified recombinant protein (amino acids 1-313) supplied by S. Gerondakis (Wajer and Eliza Hall Institute, Melbourne, Australia).

DNase I hypersensitive site analysis. DNase I hypersensitive sites (DH) sites in C11STH cells, HUVEs, Jurkat cells, and peripheral blood T lymphocytes were assayed as described 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, and
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 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
determined as previously described by transfecting 20 μg of each plas-
mid construct into 1 × 10^6 C11TH cells by the diethylaminoethyl
(DEAE) dextran procedure^4^ and measuring luciferase expression^5^
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 C11TH cells were incubated with 100 U/mL TNF-α in the
presence or absence of 1 μmol/L CsA. After 4 hours’ activation,
^51Cr-labeled neutrophils (9 × 10^5 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 ^51Cr.

E-selectin and VCAM-1 expression. Cell surface expression of
E-selectin and VCAM-1 on C11TH cells was determined by flow
zymetric (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 path-
ways known to activate NFAT in T cells. To this end, we
stimulated C11TH cells and passaged HUVEs with a com-
bination 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 Ca^{2+}
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
C11TH cells using the GM-CSF enhancer GM430 se-
quence as a probe for NFATp/c binding (lefthand panel in
Fig 1) and the human IL-2 gene distal NFAT site as a probe
for NFAT (righthand 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 indepen-
dently of AP-1.

Significantly, nuclear extracts from HUVEs and C11TH
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 inhib-
ited 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 C11TH
and HUVE extracts as antibodies raised against either recom-
binant NFATp or to an NFATp peptide specifically inhibited
formation of or super-shifted NFATp-like complexes as ef-
ciently as they did with Jurkat cell extracts (Fig 2). Addition
of a 5- to 20-fold greater amount of oct-1 antiserum had
little effect on NFATp complex formation with the endothe-

delial cell nuclear extracts. As the 67.1 NFATp antibody was
raised against a peptide not conserved in NFATc, there ap-
peared to be little or no NFATc present in either the endothe-

delial cell or the Jurkat cell extracts employed in this study.

NFATp induction in endothelial cells may require a Ca^{2+}
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 NF-kB 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 Ca^{2+} 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 RNAase pro-
tection assays of GM-CSF mRNA in HUVEs and C11TH
cells. Just as we observed for GM-CSF protein synthesis, GM-
CSF mRNA was strongly induced in both HUVEs and
C11TH 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 C11TH 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 ex-
pression by pathways that do not use Ca^{2+}. 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 DNase I hypersensitive
site in endothelial cells. In T cells, the GM-CSF enhancer
exists as an inducible CsA-sensitive DNase I 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 = 0.0006) in the TNF-α-induced neutrophil adherence.
GM-CSF AND E-SELECTIN GENE EXPRESSION

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. VCAM-1 plays a major role in the adherence of monocytes and lymphocytes to endothelial cells.

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 (PMA/II) (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. On inclusion of increasing amounts of CsA, we observed up to a 40% decrease in the cell surface expression of E-selectin expression in C11STH cells activated by either TNF-α, TNF-α plus IFN, or PMA and A23187 (Fig 7). CsA reduced the expression obtained with PMA and A23187 to a level equivalent to that obtained with PMA alone. Furthermore, activation of E-selectin expression in the presence of PMA alone was essentially resistant to the effects of CsA.

Interestingly, CsA also did not suppress the component of TNF-α-inducible E-selectin expression that was resistant to forskolin. The effects of CsA on E-selectin expression appeared to be specific, as CsA had no effect on the induction of VCAM-1 expression by any of the above agents (data not shown).

A CsA-sensitive DNAseI hypersensitive site exists in the E-selectin promoter. To search for regulatory elements that might account for the CsA-sensitivity of E-selectin gene expression, we screened the E-selectin locus for CsA-sensitive DH sites. For this purpose we reprobed the filter used in Fig 5 with an intragenic E-selectin probe.

DNAseI digestion of the E-selectin chromosomal locus produced additional 3.0- and 6.5-kb subfragments of the 9-kb EcoRI fragment, indicating that two DH sites existed upstream of the E-selectin gene in activated endothelial cells (Fig 8). One DH site near the origin of transcription was induced by either TNF-α or a combination of PMA and A23187 in both C11STH cells (Fig 8A) and HUVEs (Fig 8B) and has also recently been detected by others. 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.
Fig 5. Mapping of DH sites from an EcoRI site 6.8 kb upstream of the GM-CSF gene in endothelial cells. 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/II) of 0.1 μmol/L CsA. (A) shows DH sites in C11STH cells. Note that these sites are not detected in the absence of DNase1 digestion (control DNA). P represents a DH site in the proximal region of the promoter, whereas E represents a DH site located in the center of the enhancer 2.8 to 3.0 kb upstream of the transcription start site.

Fig 6. Activation of GM-CSF enhancer/promoter luciferase reporter gene plasmids in endothelial cells. Luciferase gene plasmids containing either the GM-CSF promoter (pGMlucl) or the GM-CSF enhancer linked to the promoter (pGMELuc) were transfected into C11STH cells and stimulated for 9 hours with 20 ng/ml PMA and 2 μmol/L A23187 in the presence (PMA/I/CsA) or absence (PMA/II) of 0.1 μmol/L CsA. Luciferase activities were expressed as a percentage of the values obtained with stimulated pGME and represent the mean of nine experiments. Error bars represent the standard deviation.

p50, and NF-κB p65 to the best characterized NF-κB site in the E-selectin promoter (PD146), 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.59

As anticipated from earlier studies of T-cell nuclear extracts,21 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 potential

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Adhesion (cpm x 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.
Fig 7. CsA suppresses induction of E-selectin expression in endothelial cells. E-selectin expression was induced in C11STH 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 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 or absence of 0.1 μmol/L CsA or with 100 U/mL TNF-α. (A) shows DH sites in C11STH 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.

DISCUSSION

To gain new insights into processes that regulate inflammation, we focused on the endothelium, which orchestrates...
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 activator 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-KB sites, which represent 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 promoter may itself be regulated by CsA as it includes the sequence TGGACCGCATGACTCTTA 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.
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GW Cockerill, AG Bert, GR Ryan, JR Gamble, MA Vadas and PN Cockerill