Interleukin-10 Induction of Nitric-oxide Synthase Expression Attenuates CD40-mediated Interleukin-12 Synthesis in Human Endothelial Cells*

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Interleukin-10 (IL-10) is a potent anti-inflammatory cytokine in Th1 cell-mediated chronic inflammatory diseases such as, e.g. Crohn’s disease. Moreover, IL-10 has been shown to limit the progression of atherosclerosis, presumably by influencing endothelial cell function. Here we demonstrate that under pro-inflammatory conditions expression of the human IL-10 receptor gene is enhanced in endothelial cells in vitro and in vivo. Subsequent exposure to IL-10 results in an up-regulation of both endothelial nitric-oxide synthase (NOS-3) expression and activity. Gel mobility shift analyses and decoy oligonucleotide experiments suggest that this effect of IL-10 is mediated through activation of the transcription factor STAT-3 (signal transducer and activator of transcription-3). One functional consequence of IL-10 up-regulation of NOS-3 abundance in cultured endothelial cells is the attenuation of CD154-induced IL-12 p40 expression. Moreover, CD154-induced IL-12 p40 expression is enhanced after blockade of NOS-3 activity but attenuated in the presence of exogenous nitric oxide. Increased NOS-3 expression may, thus, be one mechanism by which IL-10 exerts its anti-inflammatory effects in Th1 cell-mediated chronic inflammatory diseases.

Chronic inflammatory diseases are caused and maintained by a multitude of both exogenous and endogenous factors. One subgroup of these diseases including Crohn’s disease or atherosclerosis is characterized by the activation and extravasation of T-lymphocytes predominantly of the Th1-type (Th1 cells) and expression of Th1-prototypic cytokines such as interleukin-12 (IL-12) in vivo (1, 2).

IL-10 is a functional antagonist of IL-12, blocking its expression in antigen-presenting cells such as macrophages (3). Moreover, IL-10-deficient mice have been shown to develop Th1-type chronic inflammatory diseases with age (4), and recombinant IL-10 has been employed to treat patients with Crohn’s disease (5) or psoriasis (6) with promising results. IL-10-deficient mice are also prone to develop atherosclerosis (7), and although the precise mechanism of this anti-atherosclerotic effect of IL-10 is not known, it may be endothelium-dependent (8).

In addition to professional immune cells, endothelial cells play an important role in chronic inflammatory disease, because they are required to coordinate the onset, progression, and termination of inflammation. On the one hand, they act as gatekeepers, promoting or attenuating the extravasation of leukocytes at sites of inflammation (9). On the other hand, they are capable of expressing pro-inflammatory gene products, thereby augmenting activation of the infiltrating leukocytes further (10). Whether endothelial cells promote or attenuate inflammatory responses to a large part seems to hinge on the level of nitric oxide (NO) production in these cells. NO is a highly potent anti-inflammatory agent that has been demonstrated to retard the progression of atherosclerosis (11) and to inhibit the development of other chronic inflammatory diseases (12).

Taking into account both the (presumably endothelium-mediated) capacity of IL-10 to suppress Th1 cell-mediated inflammation in vivo and the anti-inflammatory properties of endothelium-derived NO, we have addressed the question under which conditions human endothelial cells are susceptible to IL-10 signaling and whether IL-10 can exert its anti-inflammatory effects by stimulating endothelial NO synthase (NOS-3).

EXPERIMENTAL PROCEDURES

Reagents—Recombinant human IL-10 was obtained from PeproTech (via TEBU, Frankfurt, Germany). The sis-inducible element (SIE) gel shift oligonucleotide and the monoclonal antibody against signal transducer and activator of transcription-3 (STAT-3) were from Santa Cruz Biotechnology (Heidelberg, Germany). The sis-inducible element (SIE) gel shift oligonucleotide and the monoclonal antibody against signal transducer and activator of transcription-3 (STAT-3) were from Santa Cruz Biotechnology (Heidelberg, Germany). Monoclonal mouse anti-IL-10 receptor and anti-NOS-3 antibodies were obtained from Pharmingen, and monoclonal antibodies against IL-10 and IL-10 standard protein were obtained from BioSource (Nivelles, Belgium). Monoclonal antibodies against human von Willebrand factor, smooth muscle α-actin, and the secondary goat anti-mouse antibody coupled to horseradish peroxidase were obtained from Sigma-Aldrich. Secondary antibodies coupled to Alexa green and 5-(carboxy)-2′,7′-dichlorodihydrofluorescein diacetate were obtained from Molecular Probes (via MoBiTec, Göttingen, Germany). The peroxidase-coupled secondary anti-mouse antibody and the 3-amin-9-ethyl-carbazole (AEC) chromogen solution for immunohistochemistry were from DAKO, Hamburg, Germany. Hematoxylin was from Merck, and the Aquamount sealing medium was purchased from Gur, Hanau, Germany. Culture media, fetal bovine serum, Hanks’ balanced salt solution, and dispase were from Invitrogen, and endothelial cell growth supplement (ECGS plus heparin) was from PromoCell (Heidelberg).
IL-10 and NOS-3 Expression

TABLE I

| Gene product | Sequence | Annealing temperature |
|--------------|----------|-----------------------|
| VCAM-1       | Forward 5'-CATGACCTGTTCCAGGAGG-3' | 63 °C |
|              | Reverse 5'-ATTCTACGAGGCCACACTC-3' | 58 °C |
| GAPDH        | Forward 5'-TACCATCCTTCGAGGAGG-3' | 58 °C |
|              | Reverse 5'-GTGCTTCACCACTCTTGGA-3' | 58 °C |
| IL-10        | Forward 5'-GAGAACGCTCTGACCACCTCC-3' | 58 °C |
|              | Reverse 5'-AGTCGCCACCCTGATCTCAGGGA-3' | 58 °C |
| IL-10 receptor | Forward 5'-GGAGCCCATTCACAAAATCAGCTC-3' | 59 °C |
|              | Reverse 5'-CAAGGTTGAATAACTCCTGGTGGT-3' | 62 °C |
| IL-12 p40    | Forward 5'-GTTATCTCACCATTCTACCTTC-3' | 56 °C |
|              | Reverse 5'-TCTGGGCTTCTTCGTTGTCGTC-3' | 56 °C |
| MCP-1        | Forward 5'-CCATCTCCCTTACCAGCATGAGAAGT-3' | 58 °C |
|              | Reverse 5'-TCCCTGATTGGTGGAGGTGAG-3' | 56 °C |
| NOS-2        | Forward 5'-TACATCGAACGATAATC-3' | 55 °C |
|              | Reverse 5'-CTGATCAAATGTCGACGAGC-3' | 58 °C |
| NOS-3        | Forward 5'-GGAGCTGTGGTGGACCCCACT-3' | 58 °C |
|              | Reverse 5'-CCACGTGCTAATCTCAGAC-3' | 58 °C |
| rpl32        | Forward 5'-GTTTACATCGGCAAGACTGCAG-3' | 60 °C |
|              | Reverse 5'-AGCGTACATGACAGTGTCCTAC-3' | 60 °C |

Western Blot Analysis—Western blot analysis was performed according to standard procedures (14) using polyvinylidene difluoride membranes. Loading and transfer of equal amounts of protein in each lane was verified by reprobing the membrane with a monoclonal anti-β-actin antibody (14). Densitometric analysis of protein amounts relative to β-actin was performed using the One-DScan Gel analysis software (Scananalytics, Billerica, MA) where appropriate.

Enzyme-linked Immunosorbent Assay—Enzyme-linked immunosorbent assay was performed exactly as described previously (13). In brief, cell culture supernatants were collected at the times indicated, centrifuged for 5 min at 3000 rpm to pellet cell debris and CD154-positive myeloma cells, and frozen in liquid nitrogen until analysis.

Immunohistochemistry—Immunohistochemistry was performed as described previously (13). In brief, formaldehyde-fixed ileum biopsies derived from patients with Crohn’s disease or the corresponding biopsies of non-inflamed ileum resected from tumor patients were embedded in paraffin blocks of which 3-μm-thick sections were cut. After paraffin removal samples were blocked for nonspecific binding. The primary mouse monoclonal anti-IL-10 receptor antibody was diluted 1:50 and incubated with the sections for 2 h at room temperature. After extensive rinsing a peroxidase-coupled secondary antibody was added and incubated for 30 min at room temperature. Thereafter, the sections were rinsed again, incubated for 20 min at room temperature with the ABC chromagen solution, counter-stained with hematoxylin, and finally, mounted with sealing medium.

Nitrite Determination—Nitrite was measured as an estimate of NO production by a modified Griess reaction (16). To obtain IL-10 effects independent from vitamin D3, the cultured endothelial cells were pre-incubated with a combination of cytokines (60 units/ml IL-1 β, 1000 units/ml TNF-α, and 1000 units/ml IFN-γ) for 12 h to induce IL-10R expression. After this time, the cells were washed twice with culture medium and then exposed to IL-10 (2 μg/ml) and/or to the same combination of cytokines as described before. Cell culture supernatants were collected 48 h post-cytokine exposure and assayed for their nitrite content as described previously (16).

Determination of Superoxide Formation—Generation of superoxide in the cultured endothelial cells was estimated as described previously (17). Phorbol 12,13-dibutyrate was used as a positive control and changes in the cells capacity to generate superoxide in response to acute (1–10 min) and prolonged (6–12 h) exposure to IL-10 (2 μg/ml) or a combination of pro-inflammatory cytokines (60 units/ml IL-1 β, 1000 units/ml TNF-α, and 1000 units/ml IFN-γ) were monitored.

Statistical Analysis—Unless indicated otherwise, results are expressed as the means ± S.E. of n observations with cells obtained from different umbilical cords. One sample test with two-sided p value or one-way analysis of variance followed by Dunnett post-test were used as appropriate to determine statistically significant differences between the means and/or the means and control with p < 0.05 considered significant.
RESULTS

Induction of IL-10 Receptor Expression by Various Pro-inflammatory Stimuli—To confirm that the cultured endothelial cells are capable of responding to IL-10 treatment, first IL-10R1 expression was analyzed. Although clearly detectable by RT-PCR analysis, little IL-10R1 mRNA was present in quiescent cells. However, exposure to certain pro-inflammatory cytokines such as cell-bound CD154 or IFN-γ for 6 h resulted in a distinct up-regulation of IL-10R1 mRNA expression (Fig. 1A). This effect was even more pronounced when the two stimuli were combined. Also, the combination of IFN-γ with TNFα and/or IL-1β induced IL-10R1 mRNA expression to a similar extent. On the protein level, CD154 or IFN-γ also triggered an increase in IL-10R1 expression that was comparable with the change in mRNA expression (Fig. 1B). Moreover, incubation of the cultured endothelial cells with vitamin D3 (10 nmol/liter), previously characterized to induce IL-10R1 mRNA in human keratinocytes (18), revealed a transient increase both in IL-10R1 mRNA and protein (Fig. 1).

Unlike the changes in IL-10R1 expression, there was no appreciable release of the cytokine itself from the cultured endothelial cells according to enzyme-linked immunosorbent assay irrespective of the chosen experimental conditions (quiescent or cytokine-stimulated cells). IL-10 mRNA expression, on the other hand, could be detected by RT-PCR analysis in some batches of cells at a very low level (not shown).

To monitor IL-10R1 expression in vivo, intestinal biopsies from patients with Crohn’s disease were compared with those from non-inflamed controls by immunohistochemistry. Whereas endothelial cells in arteries were devoid of IL-10R1 immunoreactivity in both types of specimens (not shown), both arteriolar and venous endothelial cells stained positive for IL-10R1 but only in biopsies of patients with Crohn’s disease (Fig. 2). Interestingly, medial smooth muscle cells also revealed IL-10R1 immunoreactivity that was increased under pro-inflammatory conditions (Fig. 2). Both these findings were confirmed at the mRNA level in human cultured smooth muscle cells by RT-PCR analysis (not shown).

IL-10 Up-regulation of NOS-3 Expression—To better visualize the effects of IL-10 on endothelial cell gene expression, in all subsequent experiments IL-10R1 expression was first up-regulated by exposing the cells to vitamin D3 for 9 h. This preincubation period was chosen based on the time course of IL-10R1 expression in three individual batches of endothelial cells (not shown). The reason for using vitamin D3 to up-regulate IL-10R1 expression was that vitamin D3, in contrast to the aforementioned cytokines, neither affected the expression of pro-inflammatory gene products such as the chemokine monocyte chemoattractant protein-1 or the adhesion molecule vascular cell adhesion molecule-1 nor that of the NO synthase isoforms NOS-3 or NOS-2 (not shown).

Next, the effects of IL-10 on endothelial cell gene expression were analyzed. Exposure of the cultured cells to IL-10 (2 ng/ml) resulted in a time-dependent increase in both NOS-3 mRNA (Fig. 3A) and protein (Fig. 3B). Expression of NOS-2, on the other hand, was not detected by RT-PCR analysis in these cells, irrespective of the chosen experimental conditions (not shown).

To explore if IL-10 is capable of functionally counteracting cytokine down-regulation of NOS-3 expression in the cultured endothelial cells (cf. Ref. 19) independently from vitamin D3, nitrite concentrations in the conditioned media were determined as a means of NOS-3 activity. To this end, the cells were preincubated for 12 h with IL-1β (60 units/ml), TNF-α (1000 units/ml), and IFN-γ (1000 units/ml; Ref. 19) to induce IL-10R1 expression. Thereafter, they were exposed for 48 h to IL-10 (2 ng/ml), the cytokine combination, or both. Although IL-10 clearly enhanced NOS-3 activity, this was reduced in the presence of the cytokines but maintained after exposure to both IL-10 and the combination of cytokines (Fig. 3C). In contrast to NOS-3 activity, the cytokine combination affected neither basal nor phorbol ester-stimulated superoxide formation in the cultured endothelial cells, as judged by 5-(carboxy)-2’,7’-dichlorodihydrofluorescein diacetate fluorescence analysis at differ-
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IL-10 Up-regulates NOS-3 Expression via STAT-3—Because IL-10 has been reported to exert its genomic effects primarily by activating the transcription factor STAT-3, next, nuclear translocation of STAT-3 was monitored in IL-10-stimulated cells. Gel mobility shift analysis using either an SIE (STAT family) or the S3 oligonucleotide mimicking the putative STAT-3 binding site in the human nos-3 promoter demonstrated increased binding of a nuclear protein that was identified as STAT-3 according to supershift analysis (Fig. 4A). Moreover, neutralization of the transcription factor by employing a decoy oligonucleotide encompassing the putative STAT-3 binding site in the nos-3 promoter virtually abolished IL-10-induced NOS-3 expression, whereas the corresponding control oligonucleotide revealed no significant effect (Fig. 4B).

Attenuation of CD154-induced IL-12 p40 Expression—To assess the functional consequences of IL-10-induced NOS-3 expression, its effect on expression of the p40 subunit of IL-12 was investigated. Exposure of the cultured endothelial cells to CD154-expressing mouse myeloma cells, but not P3×63Ag8.6B control cells (cf. Ref. 13), resulted in a significant induction of IL-12 p40 mRNA and protein. This was inhibited by ~60% on the mRNA and by ~50% on the protein level in cells pretreated with vitamin D3 and subsequently IL-10 (Figs. 5, A and B).

To confirm that this inhibitory effect was indeed brought about by IL-10 up-regulation of NOS-3 expression, hence increased NO formation, the sensitivity of CD154-induced IL-12 p40 expression to NO was studied. Blockade of endogenous NO synthesis with the selective NOS-3 inhibitor (as compared with NOS-2; cf. Ref. 20), Nω-nitro-L-arginine (0.1 mmol/liter), 1 h before CD40 stimulation clearly augmented IL-12 p40 expression, whereas this was significantly attenuated upon exposure...
of the endothelial cells to the NO donor (cf. Ref. 21), CAS-1609 (10 µmol/liter; Fig. 6). Moreover, the decrease in CD154-induced IL-12 p40 expression in IL-10-treated endothelial cells was blunted upon exposure to G-nitro-L-arginine (0.1 mmol/liter, 1 h before CD40 stimulation) (Figs. 5 and 6). To exclude any source for NO other than NOS-3, mRNA derived from endothelial cells exposed to IL-10 and the CD154-expressing mouse myeloma cells was analyzed for NOS-2 expression, again with negative results (not shown).

DISCUSSION

The data presented here provide three novel findings; (i) expression of the il-10r1 gene in human endothelial cells is cytokine-inducible so that IL-10 receptor expression in these cells is likely to be up-regulated during chronic inflammation such as, e.g., in Crohn’s disease or atherosclerosis; (ii) IL-10 up-regulates NOS-3 expression in IL-10 receptor expressing endothelial cells presumably through activation of STAT-3; and (iii) the increase in NO formation associated with this change in gene expression enables endothelial cells to control pro-inflammatory reactions such as CD154 induction of IL-12 p40 gene expression. Moreover, this increase in the NO-synthesizing capacity of the endothelium may constitute a critical aspect of the anti-inflammatory action of IL-10 in Th1 cell-mediated chronic inflammation.

The Experimental Model—Although various pro-inflammatory cytokines were capable of up-regulating IL-10 receptor expression in the cultured endothelial cells, they were excluded from further studies due to their pronounced inhibitory effects on NOS-3 mRNA expression (19). Vitamin D3 was used instead solely to enhance the endothelial cell response to IL-10 stimulation. Although derivatives of vitamin D3 are thought to have anti-inflammatory potential (22), vitamin D3 per se neither affected NOS-3 expression nor that of other gene products of interest such as monocyte chemoattractant protein-1, vascular cell adhesion molecule-1, or NOS-2 in the cultured endothelial cells.

To functionally evaluate one possible consequence of the IL-10-induced increase in NOS-3 expression, CD154 induction of IL-12 p40 expression was chosen. Leukocytes contaminating the cultured endothelial cells as a source of IL-12 p40 mRNA or protein could effectively be ruled out due to the absence of CD45-positive cells (cf. Ref. 13).

The IL-10 Receptor in Endothelial Cells—The IL-10 receptor complex, a member of the IFNγ receptor family, consists of two polypeptides, the principal IL-10 receptor, i.e., IL-10R1, and an accessory receptor (23). Whereas the IL-10 receptor complex is constitutively expressed by T helper cells, B-cells and monocytes, other cell types do not normally express these proteins in significant amounts. Only a few reports exist in the literature providing direct evidence for IL-10R1 mRNA expression in non-immune cells such as e.g., in human keratinocytes (18). Although several effects of IL-10 on endothelial cells have been reported (8), this to our knowledge is the first demonstration of IL-10R1 expression in human endothelial cells. Despite being detectable in the cultured endothelial cells also under basal conditions, IL-10R1 protein expression was markedly up-regulated in response to various pro-inflammatory stimuli such as INFγ, TNFα, or cell-bound CD154, which are highly abundant at sites of inflammation as well as in atherosclerotic lesions.
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(24). In this context, IL-10R1 immunoreactivity was exclusively detected in endothelial cells lining the submucosal blood vessels of the inflamed ileum from patients with Crohn's disease but not in control biopsies from patients with intestinal carcinoma. These findings imply that endothelial cells become more responsive to IL-10 during an inflammatory episode via de novo synthesis of the IL-10 receptor.

Transcriptional Regulation of the nos-3 Gene by IL-10—Combination of gel mobility (super)shift analysis and the decoy oligonucleotide technique (cf. Refs. 12 and 25) revealed that IL-10 up-regulates NOS-3 expression in the cultured endothelial cells via activation of STAT-3. Although a binding site for STAT-3 could be predicted by sequence analysis of the human nos-3 gene promoter, this finding nonetheless is surprising because this transcription factor typically is involved in the expression of pro-inflammatory gene products (26). That IL-10 itself potentially displays such an ambiguity in its biological effects was demonstrated in an additional series of experiments discussed below.

Functional Consequences of IL-10-induced NOS-3 Expression—The role of NO in chronic inflammation is somewhat controversial. Whereas excessive NO formation due to induction of NOS-2 in combination with an increased generation of superoxide might be responsible for inflammation-induced tissue damage (12), potent anti-inflammatory properties have been ascribed to NOS-3-derived NO (11, 27). However, in the early phase of an inflammatory episode the low endothelial cell NO output, which is further decreased through cytokine-mediated down-regulation of NOS-3 expression, may not be sufficient to effectively limit the pro-inflammatory response. The IL-10-induced increase in NOS-3 expression may thus counterbalance the inhibitory effect of the cytokines and restore endothelial cell NO formation to normal levels. Our findings of a maintained NOS-3 expression, i.e. NO output, in endothelial cells exposed to both a combination of pro-inflammatory cytokines and IL-10 (and without pretreatment of the cells with vitamin D3 to up-regulate IL-10 receptor expression) strongly supports this hypothesis.

The bioavailability of NO is limited by the amount of superoxide concomitantly formed, for the two radicals rapidly neutralize each other to yield the biologically inactive nitrate (28). Pro-inflammatory cytokines such as, e.g. TNFα, may enhance superoxide formation in endothelial cells, which in addition to the decrease in NOS-3 expression could offset the stimulatory effect of IL-10 on the formation of biologically active NO. In our hands, however, these cytokines did not reveal any appreciable effect on endothelial cell superoxide formation, so that our hypothesis of a restoration of NO bioavailability in endothelial cells exposed to IL-10 at sites of inflammation, thereby limiting the expression of other pro-inflammatory gene products in an autocrine/paracrine manner, still stands.

The marked attenuation of CD154-induced IL-12 p40 expression after IL-10 up-regulation of NOS-3 expression is one example for such an effect. CD154-induced IL-12 p40 expression was chosen as a “readout” for NOS-3-mediated IL-10 effects on pro-inflammatory gene expression for the following reasons; (i) CD40 stimulation, in contrast to the pro-inflammatory cytokines employed, does not affect NOS-3 expression itself; (ii) IL-12 p40 expression in primary human cultured endothelial cells is exclusively induced via CD40 stimulation (13); (iii) IL-12 plays an important role in the onset and maintenance of Th1-cell mediated (chronic) inflammatory diseases including atherosclerosis (29); and (iv) IL-12 synthesis in antigen-presenting cells is the prime target of the anti-inflammatory actions of IL-10 in these diseases (3).

The inhibitory effect of IL-10 on IL-12 p40 expression was mimicked by exposing the cultured endothelial cells to exogenous NO and effectively lifted in cells where NOS-3 activity had been blocked. IL-10 attenuation of IL-12 expression in endothelial cells, thus, seems to be critically dependent on the increase in NOS-3 expression and, hence, activity. However, two questions remain; (i) Why does basal NO formation (blocked by Nω-nitro-l-arginine) only moderately affect IL-12 p40 mRNA expression, whereas NOS-3 blockade in endothelial cells previously exposed to IL-10 results in a super-induction of IL-12 p40 mRNA expression?

This observation, although puzzling initially, may be explained by the ambivalent nature of action of IL-10. Besides its prominent anti-inflammatory effects, mainly directed against antigen-presenting cells, the cytokine also has a pro-inflammatory potential, e.g. in TH2 cell-dependent immune responses, which may be unmasked in endothelial cells through NOS-3 blockade. (ii) Why is this super-induction of IL-12 p40 expression only detectable at the mRNA level? Comparable effects have been described for other pro-inflammatory gene products, such as IL-1β or TNFα (for review see Ref. 30). Although the mechanism of this “programmed mismatch” between mRNA and protein expression is not fully understood, it is well documented that besides a decrease in mRNA stability, mRNA translation into protein can be strongly blunted with respect to these gene products. Nonetheless, the ambiguous action of IL-10 on the level of gene transcription may be of pathophysiological relevance. IL-10 may, thus, be beneficial in conditions with unrestrained endothelial NO synthesis as, e.g. in the early phase of a chronic inflammatory reaction, but without effects or even detrimental in conditions of low NO bioavailability as, e.g. in manifest inflammation (26).

Conclusions—The present findings suggest that IL-10 acts as an “endogenous brake,” limiting the development of chronic inflammatory diseases (3–6, 31) not only by directly attenuating the activity of macrophages and TH1 cells but also through the maintenance of NOS-3 expression in endothelial cells at sites of inflammation. Consequently, the administration of recombinant IL-10 constitutes a promising concept in the treatment of chronic TH1-type immunoregulatory disorders such as, e.g. Crohn’s disease (6) or psoriasis (7). However, from the experimental findings presented here, it would appear that the timing and/or duration of IL-10 treatment is crucial for its therapeutic success.

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