Clinical Trial of Doxycycline for Matrix Metalloproteinase-9 Inhibition in Patients With an Abdominal Aneurysm

Doxycycline Selectively Depletes Aortic Wall Neutrophils and Cytotoxic T Cells

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Background—Doxycycline has been shown to effectively inhibit aneurysm formation in animal models of abdominal aortic aneurysm. Although this effect is ascribed to matrix metalloproteinase-9 inhibition, such an effect is unclear in human studies. We reevaluated the effect of doxycycline on aortic wall protease content in a clinical trial and found that doxycycline selectively reduces neutrophil-derived proteases. We thus hypothesized that doxycycline acts through an effect on vascular inflammation.

Methods and Results—Sixty patients scheduled for elective open aneurysmal repair were randomly assigned to 2 weeks of low-, medium-, or high-dose doxycycline (50, 100, or 300 mg/d, respectively) or no medication (control group). Aortic wall samples were collected at the time of operation, and the effect of doxycycline treatment on vascular inflammation was evaluated. Independently of its dose, doxycycline treatment resulted in a profound but selective suppression of aortic wall inflammation as reflected by a selective 72% reduction of the aortic wall neutrophils and a 95% reduction of the aortic wall cytotoxic T-cell content (median values; $P<0.00003$). Evaluation of major inflammatory pathways suggested that doxycycline treatment specifically quenched AP-1 and C/EBP proinflammatory transcription pathways ($P<0.0158$, NS) and reduced vascular interleukin-6 ($P<0.00115$), interleukin-8 ($P<0.00246$, NS), interleukin-13 ($P<0.0184$, NS), and granulocyte colony-stimulating factor ($P<0.031$, NS) protein levels. Doxycycline was well tolerated; there were no adverse effects.

Conclusions—A brief period of doxycycline treatment has a profound but selective effect on vascular inflammation and reduces aortic wall neutrophil and cytotoxic T-cell content. Results of this study are relevant for pharmaceutical stabilization of the abdominal aneurysm and possibly for other inflammatory conditions that involve neutrophils and/or cytotoxic T cells. (***Circulation***. 2009;119:2209-2216.)

Key Words: aneurysm ■ immunology ■ inflammation ■ molecular biology ■ trials

Pharmaceutical strategies inhibiting aneurysm growth, thereby reducing the need for surgical repair, could have major advantages for patients and socioeconomically.[1,2] An abundance of matrix metalloproteinase-9 (MMP9) in growing abdominal aortic aneurysms (AAAs),[3] along with the observation that disruption of MMP9 gene prevents AAA formation,[4] led to the notion that MMP9 is critically involved in AAA formation.[5] It was thus proposed that pharmaceutical inhibition of MMP9 could restore the balance between matrix degradation and deposition,[5] thereby reducing aneurysmal growth.[6]

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Independently of its antibiotic properties, the tetracycline analogue doxycycline has been shown to reduce both MMP9 expression and activity,[7] suggesting that doxycycline treatment may reduce aneurysmal growth. Indeed, doxycycline has been convincingly shown to prevent AAA formation in a variety of animal models,[6,8–10] and the results from 2 small clinical studies suggest that doxycycline also reduces AAA expansion in humans.[11,12] Remarkably, although the rationale behind doxycycline therapy is based on its putative effects on MMP9 expression and activity,[6] the effects of doxycycline on MMPs in the human aneurysm are unclear, with published studies[13,14] suggesting that doxycycline acts through a different mechanism.[1]

In vitro studies characterize doxycycline as a pleiotropic antiinflammatory and immunomodulatory agent.[7] However, the relevance of this well-tolerated compound[15] for modulat-

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ing inflammation in human disease in general and AAA in particular is unclear. In this prospective clinical trial, we systematically examined the effect of 3 pharmacologically relevant doses (low [50/d], regular [100 mg/d], or high [300 mg/d]) of doxycycline on MMP9 expression and inflammatory processes in the aneurysmal wall of patients scheduled for elective open aneurysm repair. Evaluation of the effect of doxycycline treatment showed that MMP9 was reduced on the protein but not on the mRNA level. Because aortic neutrophils carry large amounts of preformed MMP9 protein and lack de novo protein synthesis,[16,17] we hypothesized that the observed reduction of MMP9 protein may relate to a change in the aortic content of neutrophils. The present study shows that a brief period of 2 weeks of doxycycline treatment significantly reduces the aortic content of neutrophils and of cytotoxic T cells, another cell type considered crucial for the process of AAA formation.[18–21] Based on these findings on the cellular level, a molecular analysis on the level of relevant cytokines and transcription factors[22] was performed next.

Methods

The present randomized, dose-ranging study was performed in patients awaiting open aneurysm repair. The trial included 3 treatment groups and 1 nontreated (control) group. Randomization was performed by block randomization (blocks of 20 patients). Randomization lists were generated by the Leiden University Medical Center Department of Medical Statistics, and randomization was performed by the Leiden University Medical Center pharmacy. The study was performed in an investigator-blinded fashion. Planned open aneurysmal repair of an infrarenal aortic aneurysm was the primary inclusion criterion. Decision for open repair was based on anatomic (eg, neck, elongation) and patient (age) characteristics and patient preferences. Patients with kidney dysfunction (estimated clearance <30 mL/min), chronic inflammatory disease, or (suspected) so-called inflammatory aortic aneurysms were excluded from participation in the study. Before randomization, 2 patients were excluded because of a suspected inflammatory aneurysm. Between November 2001 and April 2005, a total of 60 patients from 4 centers in the Netherlands were randomized to receive doxycycline (at a dose of 50, 100 or 300 mg once daily) or no medication (control group) in the 2 weeks preceding open elective aneurysm repair (AAA diameter >5.5 cm). In 2 patients in the low-dose (50 mg/d) doxycycline group, the operation had to be postponed for logistical reasons; hence, 13 patients were therefore evaluated in this group. Medication was started 14 days before the planned operation, and the last dose was taken in the evening before surgery. Patient compliance was assessed by counting the medication (pills) at the end of the treatment period. This multicenter study was first approved by the Leiden University Medical Center Institutional Review Board; subsequent approval was obtained from the medical ethics committees of the other participating centers as required by Dutch law. Informed consent was obtained from each patient.

Immediately after opening of the aneurysm sac and removal of the adhering thrombus, an aortic wall tissue sample was obtained from the anterior-lateral aneurysm wall at the maximum diameter of the aneurysm. Wall samples were immediately halved; one half was snap-frozen in CO_2-cooled isopentane or liquid nitrogen and stored at −80°C for later analysis. The other half was fixed in formaldehyde (24 hours), decalcified (Kristensen's solution, 120 hours), and paraffin embedded for histological analysis. All analyses were performed in an investigator-blinded fashion.

RNA Extraction and mRNA Analysis

Total RNA extraction was performed with RNAzol (Campro Scientific, Veenendaal, the Netherlands) and glass beads according to the manufacturer's instructions. Copy DNA was prepared with the A3500 kit (Promega, Leiden, the Netherlands), and quantitative real-time polymerase chain reaction analysis was performed for MMP9, MMP12, cathepsin K, BLIMP-1, MAD-4, Ig linker protein, and IgG heavy chain on the ABI-7700 system (Applied Biosytems, Nieuwerkerk a/d IJssel, the Netherlands) with established primer/probe sets (Assays on Demand, Applied Biosystems) and Mastermix (Eurogentec, Seraing, Belgium). Analyses were performed according to the manufacturer's instructions and as previously reported.[23] GAPDH expression was used as a reference and for normalization.

Tissue Homogenization for Protein Analysis

Aortic wall tissues were pulverized in liquid nitrogen and homogenized in 2 volumes of lysis buffer (10 mmol/L Tris, pH 7.0; 0.1 mmol/L $CaCl_2$; 0.1 mol/L NaCl; 0.25% [vol/vol] Triton X-100). This protocol releases both soluble and membrane-bound proteins. Samples were subsequently centrifuged at 10 000g for 15 minutes at 4°C, snap-frozen in liquid nitrogen, and stored at −80°C until use. Protein content in homogenates was determined with a bicinchoninic acid protein assay kit (Pierce, Rockford, Ill).

Immunohistochemistry

Immunohistochemistry was performed with deparaffinized, ethanol-rehydrated tissue cross sections (thickness, 4 μm) as reported previously.[23] Cross sections were incubated overnight with polyclonal antibodies specifically staining human myeloperoxidase (rabbit polyclonal; 1:4000 dilution; DAKO, Heverlee, Belgium), CD4 (clone 1F6; 1:15 dilution; DAKO), CD8 (clone 4B11; 1:200 dilution; Novocastra, Newcastle, UK), CD20 (clone L26; 1:1000 dilution; DAKO), CD68 (clone KP6; 1:400; DAKO), and CD138 (clone B-B4; 1:1000 dilution; Serotec, Oxford, UK). Conjugated biotinylated anti-goat or rabbit anti-IgG was used as a secondary antibody. Sections were developed with Nova Red (Vector Laboratories, Burlingame, Calif) and counterstained with Mayer hematoxylin, allowing morphological analysis. Specificity of the antibody staining was confirmed by omitting the primary antibody and by isotype controls.

Specific Immunocapture MMP Activity Assays

Because of the rapid inactivation of active enzymes by endogenous inhibitors released after cell destruction by homogenation, direct measurement of active MMPs in aortic tissue homogenates is not feasible.[23] Therefore, MMP activities were measured only after in vitro activation (p-aminophenylmercuric acetate) of captured latent proenzymes. Activity was determined in established MMP8 and MMP9 immunocapture protease activity assays (Amersham Biosciences, Buckinghamshire, UK).

Western Blot Analysis

Western blot analyses was performed essentially as described previously[22] with antibodies specific for the human forms of perforin (sc-7417; Santa Cruz Biotechnology, Heerhugowaard, the Netherlands), granzyme A (M1791; Sanquin, Amsterdam, the Netherlands), p65-nuclear factor (NF)κB (nonactive conformation; sc-8008), p65-NFκB (active conformation of p65-NFκB; Chemicon, Billerica, Mass; MAB3026), c-Jun (sc-45), phospho(Ser73)-c-Jun (sc-7981), C/EBPα (sc-9315), C/EBPβ (sc-150), C/EBPδ (sc-636), and STAT3 (sc-7179) (all Santa Cruz Biotechnology); phospho-STAT3 (Epitomics PS727, Huissen, the Netherlands); and β-actin (sc-1615; Santa Cruz Biotechnology).

Corresponding secondary antibodies were obtained from Santa Cruz Biotechnology (donkey anti-goat) and Pierce (Etten-Leur, the Netherlands; goat anti-rabbit and goat anti-mouse). Immunoblots were visualized and quantified with the Super Signal West Dura Extended Duration Substrate (Perbio Science, Etten-Leur, the Netherlands), LabWorks 4.6 software, and a luminescent image workstation (UVP, Cambridge, UK). Protein expression levels were standardized for β-actin, and separate anti–β-actin immunoblots were performed for each sample.

Table 1.   Patient Characteristics

	Control AAA	Doxycycline, mg			P
		50	100	300	
Evaluable patients, n	15	13	15	15	NS
Mean age (range), y	74.8 (69–84)	72.7 (62–85)	74.1 (50–88)	72.1 (58–87)	NS
Mean AAA diameter, cm	6.7	6.5	6.3	6.7	NS
Mean time between diagnosis and surgery, mo	6	7	5	4	
Female sex, n	1	2	2	3	NS
Current smoker, n	6	6	7	5	NS
Statin use, n	1	1	1	2	NS
Antihypertensives, n	8	7	8	7	NS
Antiplatelet therapy, n	10	8	8	8	NS

Multiplex Assay and ELISAs

Aneurysm wall interleukin (IL)-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17A, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage–CSF, interferon-γ, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1β, and tumor necrosis factor-α protein levels were determined by the 17-Plex panel for multiple cytokines (Bio-Rad Laboratories BV, Veenendaal, the Netherlands). All analyses were performed in a single run. Detection thresholds for the above cytokines were <0.5 pg/mL (IL-1, IL-2, IL-5, IL-6, IL-7, IL- IL-8, IL-10, tumor necrosis factor-α), <1 pg/mL (IL-12, IL-17A), and <5 pg/mL (IL-4, IL-13, G-CSF, granulocyte macrophage–CSF, interferon-γ, MCP-1).

Most of the IL-6, IL-8, and MCP-1 levels exceeded the upper detection limit of the 17-Plex panel and were reevaluated in separate ELISAs (IL-6 and IL-8: PeliKane Compact Kit, Sanquin, Amsterdam, the Netherlands; MCP-1: Quantikine Kit, R&D Systems, Abingdon, UK).

Statistical Analysis

Power calculation for this study was based on data published in Reference 13. In that publication, doxycycline therapy reduced relative MMP9 mRNA expression from 7.2 (SE, 3.1) to 1.3 (SE, 0.5) units. The present study was designed to have a 90% power to detect a 50% reduction of MMP9 mRNA levels at a significance level of 0.05.

All values are expressed as means (SD) for normally distributed data or medians [interquartile range] for nonnormally distributed data (skewed). The level of statistical significance was dictated by Bonferroni-Holm's correction (33 variables).

One-way ANOVA for normally distributed data or the Kruskal–Wallis test for nonnormally distributed data did not indicate a difference between the 3 doxycycline doses for all variables studied (all $P>0.16$; detailed data available from the corresponding author on request). To increase the power of the study, all doxycycline-treated individuals were therefore evaluated as a single group.

The sequence of our analyses was as follows. We first analyzed the effect of doxycycline on MMP9 mRNA and protein levels. Because of the selective reduction of MMP9 protein only, we analyzed putative changes in the aortic wall neutrophil content. On observing an effect on neutrophil content, we performed a further evaluation. This analysis showed that doxycycline treatment selectively reduces aortic wall neutrophil and cytotoxic T-cell content. On the basis of this cellular evaluation, a subsequent analysis of relevant upstream- and downstream-acting cytokines and transcription factors was performed.[20]

Differences between the control group and the doxycycline-treated groups were evaluated in 1-way ANOVA with contrasts (for the normally distributed data) or by the Wilcoxon–Mann–Whitney test in the case of nonnormally distributed continuous data. Possible associations between aortic wall IL-6 and IL-8 levels and cellular content were evaluated by Pearson's correlation. All analyses were performed with SPSS 16.0 (SPSS Inc, Chicago, Ill).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Patients

Three relevant doses of doxycycline (low [50 mg/d], regular [100 mg/d], or high [300 mg/d] were evaluated.[24] A total of 60 patients were enrolled in the study. For logistical reasons, aneurysm repair had to be postponed in 2 patients; hence, a total of 58 patients could be analyzed. Doxycycline was well tolerated, and there were no withdrawals.

Patient characteristics are shown in Table 1. All 4 groups are comparable with regard to age, sex, statin use, and AAA diameter.

Statistical analysis did not indicate a difference between the 3 doxycycline doses for all variables studied; hence, all 3 doxycycline doses were combined. Probability values are for the combined doxycycline group versus the control group.

Doxycycline and MMP9 Expression

Evaluation of the effect of 2 weeks of doxycycline therapy indicated a trend toward reduced MMP9 proenzyme levels ($P<0.00263$, NS; Figure 1A) but did not influence MMP9 mRNA expression ($P<0.206$; Table 2).

Doxycycline and the Inflammatory Responses in AAA

Because of the selective effect of doxycycline treatment on MMP9 protein levels but not on mRNA expression, we next analyzed the aortic wall neutrophil content. Figure 1B and 1C show that doxycycline markedly reduced the aortic wall neutrophil content (76% reduction of the median value; $P<0.000025$). Further evaluation of other cell types in the aorta also revealed a profound reduction of the aortic wall cytotoxic (CD8^{+}) T-cell content (96% reduction of the median value; $P<0.000001$; Figure 1C and 1D) but not on other cell types (monocytes/macrophages [CD68^{+}]; T-helper cells [CD4^{+}], B cells [CD20^{+}], and plasma cells [CD138^{+}]; Figure 1E). Figure 1B and 1D illustrate that the effects of the individual doxycycline doses are equivalent.

Tissue distribution of these cells as assessed by immunohistochemistry was not influenced by doxycycline.

The decrease in neutrophil and cytotoxic T-cell count was paralleled by a reduction of granzyme A ($P<0.000010$; Figure
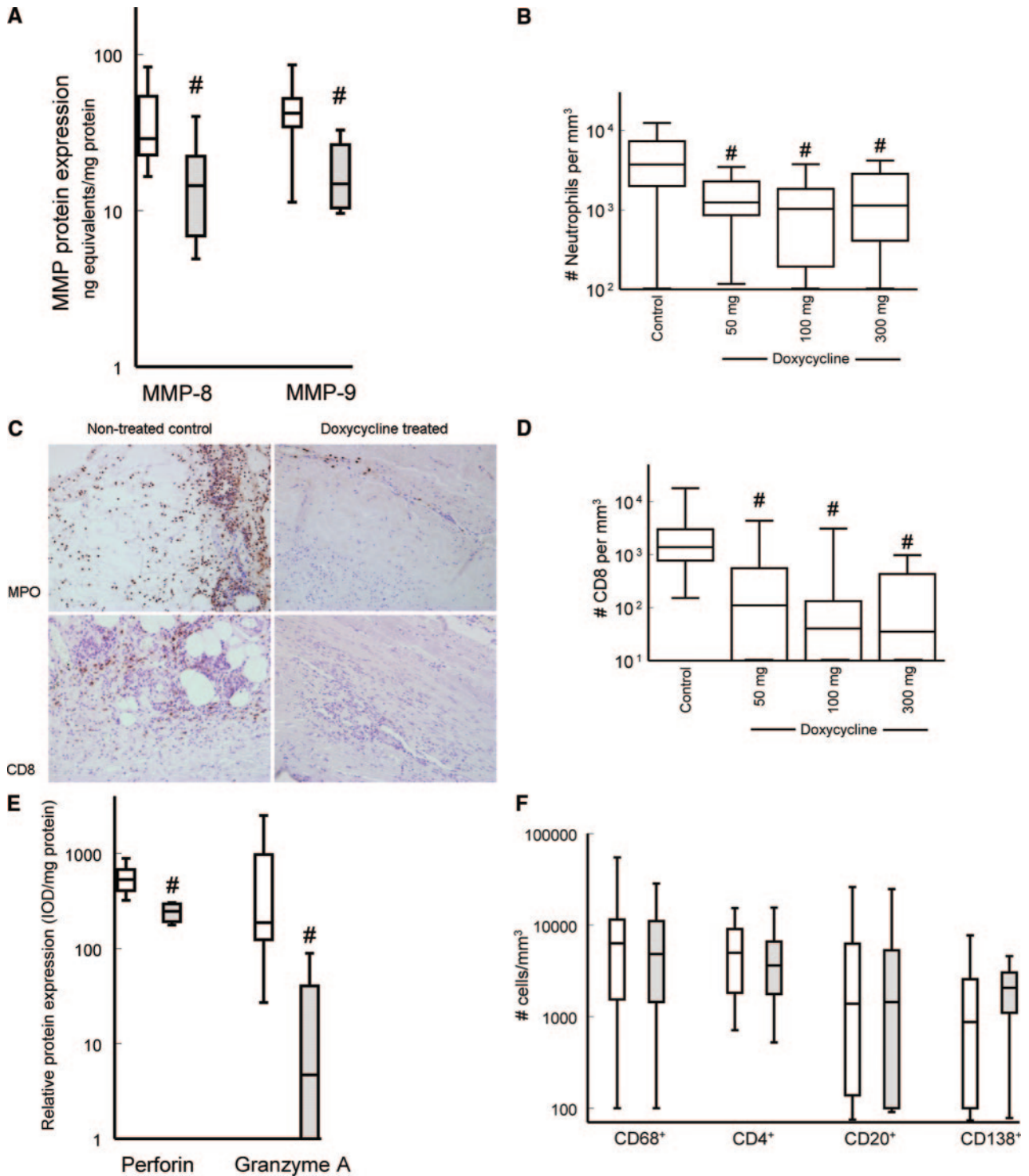
Figure 1. The effect of doxycycline on aneurysm wall leukocyte content, the neutrophil markers MMP8 and MMP9, and the cytotoxic T-cell markers perforin and granzyme A. A trend toward reduced MMP9 protein levels (proenzyme) was observed (A) after doxycycline therapy ($P<0.00263$, level of significance not reached after Bonferroni-Holm's correction). Evaluation of a putative effect on cellular content showed that treatment reduced aortic wall neutrophil (B, C; myeloperoxidase staining) and cytotoxic T-cell (C, D; CD8 staining) content ($P<0.000025$, $P<0.000001$, respectively) but not that of other cell types (E). The reduction of neutrophils and cytotoxic T cells is paralleled by a trend to reduced levels of the neutrophil marker MMP8 (A; neutrophil collagenase; $P<0.0053$, NS) and the cytotoxic T-cell activation markers perforin ($P=0.0158$, NS) and granzyme A ($P<0.00001$; F). #Significant difference between doxycycline-treated patients ■ and nontreated control subjects □.

1F), a specific marker of cytotoxic T-cell activation. The effect on the neutrophil-specific marker neutrophil collagenase (MMP8; $P<0.0053$; Figure 1A) and the cytotoxic T-cell marker perforin ($P<0.0158$) did not reach statistical significance.

mRNA levels of markers of monocyte/macrophage activation (ie, macrophage elastase [MMP12] and cathepsin K) were not affected by doxycycline therapy. Similarly, except for a small reduction of IgG heavy-chain expression, doxycycline therapy

Table 2. mRNA Expression

	Nontreated Control Subjects	Doxycycline-Treated Subjects	P*
MMP9	−1.21 (0.44)	−1.40 (0.61)	0.206 (NS)
MMP12	−2.57 (3.30 to −1.97)	−2.18 (3.28 to −1.55)	0.326 (NS)
Cathepsin K	−1.74 (0.69)	−1.85 (0.49)	0.486 (NS)
BLIMP-1	−1.64 (0.59)	−1.81 (0.48)	0.265 (NS)
MAD-4	−1.11 (0.36)	−1.11 (0.25)	0.890 (NS)
Ig linker protein	0.34 (0.39)	0.28 (0.48)	0.714 (NS)
IgG heavy chain	1.73 (0.60)	1.36 (0.48)	0.020 (NS)

NS indicates level of significance not reached after Bonferroni-Holm's correction. Log-transcript level relative to GAPDH (GAPDH=0) of markers of monocyte/macrophage activation (MMP9 and MMP12, cathepsin K) and B-cell/plasma cell activation (BLIMP-1, MAD-4, Ig linkerprotein, IgG heavy chain). Values are mean (SD) or median (interquartile range) as appropriate.

*Probability value is for the comparison of the combined doxycycline groups versus the nontreated control group.

did not influence markers of B-cell/plasma cell activity (MAD-4, BLIMP-1, and IgG linker protein; Table 2).

Effects of Doxycycline on Cytokine and Chemokine Expression

Doxycycline treatment resulted in a selective suppression of aneurysmal wall cytokine and chemokine protein levels (Table 3). IL-6 hyperexpression, a characteristic feature of AAA,[22] was reduced from median values of 462 to 148 pg/g protein (IL-6; $P < 0.00115$). The reduction of IL-8 levels (median values in the

Table 3. Effect of Doxycycline Treatment on Aneurysm Wall Cytokine and Chemokine Protein Expression

	Nontreated Control Subjects, pg/mg Protein	Doxycycline-Treated Subjects, pg/mg Protein	P*
IL-1β	4.27 (2.42–5.86)	3.50 (1.71–5.39)	0.352 (NS)
IL-2	0.28 (0.21–0.34)	0.23 (0.18–0.31)	0.389 (NS)
IL-6	462 (178–2417)	148 (63–307)	0.00115
IL-7	0.15 (0.00–0.22)	0.18 (0.11–0.29)	0.274 (NS)
IL-8	165 (92–449)	70 (34–147)	0.00246 (NS)
IL-13	1.43 (0.54–3.23)	0.57 (0.27–0.94)	0.0184 (NS)
G-CSF	6.02 (3.66–10.85)	3.48 (2.09–5.20)	0.0308 (NS)
GM-CSF	0.33 (0.00–0.69)	0.37 (0.06–0.69)	0.424 (NS)
Interferon-γ	8.16 (4.94–11.83)	5.88 (2.91–8.43)	0.109 (NS)
MCP-1	369 (211–907)	351 (190–599)	0.329 (NS)
MIP-1β	7.46 (6.10–13.48)	12.05 (6.92–18.87)	0.166 (NS)
TNF-α	0.24 (0.04–0.32)	0.17 (0.05–0.26)	0.579 (NS)

NS indicates level of significance not reached after Bonferroni-Holm's correction; GM-CSF, granulocyte macrophage CSF; MIP, macrophage inflammatory protein; and TNF, tumor necrosis factor. Values were assessed by the BioRad 17-Plex panel for multiple cytokines. IL-4, IL-5, IL-10, IL-12, and IL-17A levels were all below the detection threshold of the assay and are therefore not shown. Levels of IL-6, IL-8, and MCP-1 exceeded the upper detection threshold and were accurately quantified in separate ELISAs. Values are median (interquartile range).

*Probability value is for the combined doxycycline groups versus the nontreated control group.
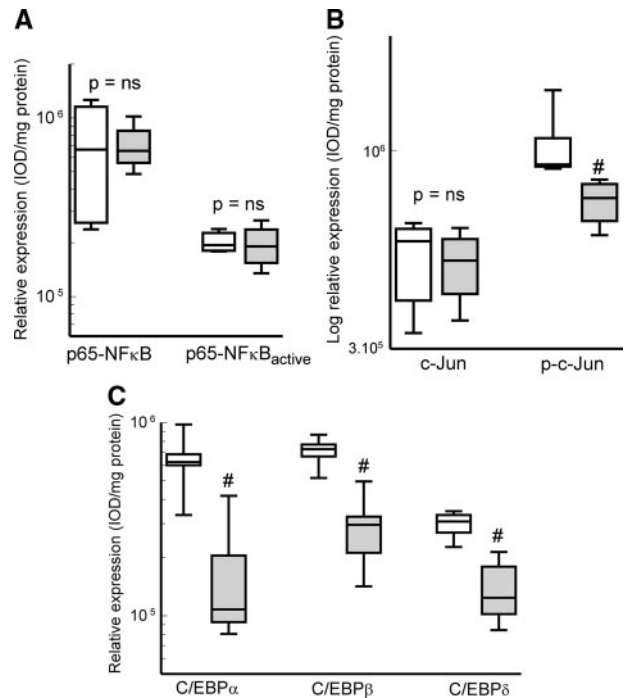

Figure 2. The effect of doxycycline on the NFκB, AP-1, and C/EBP inflammatory pathways. Doxycycline may reduce AP-1 activation (phospho-c-Jun; $P < 0.0158$, NS) and reduces C/EBPα, β, and δ expression (all $P < 0.00117$) but does not influence basal AP-1 (c-Jun) ($P < 0.905$) and NFκB ($P = 1.000$) levels and NFκB activation ($P < 0.730$). #Significant difference between doxycycline-treated patients ■ and nontreated control subjects □.

control group and the treated group, 165 and 70 pg/g protein, respectively) did not reach statistical significance ($P < 0.00246$). The strong positive relationship between neutrotactic chemokine IL-8 and aortic wall neutrophil content in the control group ($r = 0.84$, $P < 0.00016$) was not found in the doxycycline-treated group ($r = −0.037$, $P < 0.819$).

Consistent with the absence of an effect on monocyte/macrophage activation markers, doxycycline therapy did not influence the levels of inflammatory cytokines and chemokines that are associated predominantly with monocytes/macrophages, ie, IL-1β ($P < 0.352$), tumor necrosis factor-α (0.579), MCP-1 (CCL-2; $P < 0.329$), and macrophage inflammatory protein-1β (CCL4; $P < 0.166$; Table 3).

Effects of Doxycycline on Mechanistically Relevant Inflammatory Transcription Factors

Doxycycline reduced the aortic wall protein concentrations of cytokines/chemokines that are regulated by AP-1 (IL-6 and IL-8) and/or C/EBP (IL-8, G-CSF) but did not affect the level of cytokines/chemokines that are controlled predominantly by NFκB (eg, IL-1β and MCP-1). These observations suggest that doxycycline therapy specifically quenches the inflammatory transcription factors AP-1 and/or C/EBP. Evaluation of AP-1, C/EBP, and NFκB protein expression levels and their activation status (active phosphorylated c-jun for AP-1, active p65-NFκB protein confirmation for NFκB; Figure 2A and 2B) provided indications for an effect on the protein levels of all 3 C/EBP isoforms (α/β/δ; all $P < 0.00117$; Figure 2C) and possibly on the activation of the AP-1 system (phosphory-
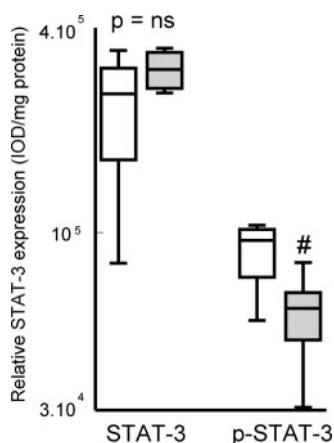
Figure 3. The effect of doxycycline treatment on basal STAT-3 levels and STAT-3 phosphorylation, the primary downstream effector pathway of IL-6 signaling. Doxycycline does not influence basal STAT-3 levels ($P=0.3428$), but a trend toward reduced STAT-3 phosphorylation (p-STAT-3; $P<0.0268$) suggests reduced IL-6 signaling. □ Indicates nontreated control subjects; ■, doxycycline-treated subjects.

lated c-jun, the major effector of AP-1; $P<0.0158$, NS). Levels of inactive and active p65-NFκB, the major effector of NFκB, were not influenced by doxycycline ($P=1.000$ and $P<0.7301$, respectively; Figure 2A).

To test whether the doxycycline-mediated suppression of IL-6 levels is sufficient to impair the IL-6 signaling pathway, we evaluated the levels of STAT-3, the primary downstream transcriptional effector of the IL-6 signaling pathway. Basal STAT-3 expression levels were comparable in doxycycline-treated and nontreated individuals ($P<0.3428$, NS), but a trend ($P<0.0268$, NS) toward reduced STAT-3 phosphorylation (ie, the activated form of this transcription factor) suggests diminished IL-6 signaling on doxycycline therapy (Figure 3).

Discussion

In this clinical trial, we demonstrate that doxycycline treatment has strong, highly selective effects on vascular inflammation in the AAA. The lowest doxycycline dose tested (50 mg/d) led to a profound reduction of aneurysmal wall neutrophil and cytotoxic T-cell content, 2 cell types considered crucial for the process of aneurysm formation.[18–21]

AAA is a common pathology and a major cause of death resulting from rupture. The hallmark pathology of AAA is a localized, chronic inflammatory response accompanied by a proteolytic imbalance.[5,22,23] Increased proteolytic activity in the disease is responsible for the increased matrix turnover and progressive weakening of the aortic wall. Prominent MMP9 expression in AAA,[3,5,6] along with resistance of MMP9 knockout mice to aneurysm formation,[4] led to the concept of MMP9 inhibition as a means of reducing AAA formation and expansion.[2] Independently of their antimicrobial properties, members of the tetracycline family have been shown to inhibit MMP9 expression[7] and activity[25] and to effectively prevent tissue injury in animal models of gingivitis, arthritis, and other inflammatory disorders.[7] On the basis of these and similar studies, it was proposed that the tetracycline analogue doxycycline may inhibit AAA progression.[2] Indeed, doxycycline has shown to forestall AAA formation

and growth in various animal models of the disease.[6,8–10] Results from 2 preliminary human studies suggest that these experimental findings may also apply to human disease.[11,12]

Remarkably, although the rationale behind doxycycline therapy is based on its putative effects on MMP9 expression and activity,[2,6] the effects are discussed controversially in published human studies.[13,14] In the first published study, Curci et al[13] evaluated the effects of preoperative doxycycline treatment (100 mg orally twice a day for 7 days) in 8 patients with an AAA and compared the results with 7 nontreated controls. They showed that doxycycline treatment resulted in a 2.5-fold reduction of MMP9 protein levels (immunoblot) and an 82% reduction of MMP9 mRNA expression (determined by a semiquantitative method, competitive real-time polymerase chain reaction). In a later placebo-controlled study, Ding et al[14] tested the effect of 1 month of doxycycline treatment (100 mg once a day) or placebo on MMP mRNA expression (real-time polymerase chain reaction) and protein levels (both ELISA and immunocapture assays) in 51 patients scheduled for open repair. Unlike the initial study by Curci et al,[13] no effect was found on MMP mRNA or protein expression.

Our findings suggest an effect of doxycycline therapy on MMP9 protein levels ($P<0.00263$), as reported by Curci et al.[13] Yet, in line with Ding et al,[14] we did not find an effect of doxycycline on MMP9 mRNA expression levels ($P<0.206$). The reducing effect of doxycycline on MMP9 protein but not on mRNA may be explained by reduced aneurysmal wall neutrophil content. Neutrophil MMP9 is produced (transcribed and translated) during neutrophil maturation in bone marrow, and the protein is subsequently stored in the secondary granules. MMP9 mRNA expression in circulating or infiltrated neutrophils is neglectable.[15,16] The absence of an effect of doxycycline on aortic wall MMP9 mRNA expression suggests that treatment does not affect MMP9 expression in other cell types (eg, macrophages). Together, these observations may suggest that the reduction of MMP9 protein can be ascribed mainly to the disappearance of aortic wall neutrophils and that the effects of doxycycline therapy relate to an antiinflammatory effect.

This study shows that the immune modulatory effects of doxycycline in AAA are profound and potentially therapeutically relevant and that these effects are highly selective; a brief period of 2 weeks of doxycycline treatment suffices to strongly reduce aortic wall neutrophil and cytotoxic T-cell content. These cellular effects were paralleled by reduced levels of cytotoxic T-cell–specific marker granzyme A. The effects on perforin ($P<0.0158$), another cytotoxic T-cell activation marker, and the neutrophil markers neutrophil collagenase (MMP8) and neutrophil gelatinase (MMP9) ($P=0.0053$ and $P=0.00263$, respectively) did not reach significance after Bonferroni-Holm's correction. Doxycycline did not affect the number and activation status of other prominent cell types in AAAs (ie, T-helper cells, monocytes/macrophages, B cells, or plasma cells),[22] showing that the immune modulatory effects of doxycycline are selective rather than universal.

In the present relatively small study, we did not observe a dose-response relationship for the parameters studied. All doxycycline-treated groups were well matched with respect to age, sex, and medication. We minimized well-known

confounding factors that may affect bioavailability. For example, we specifically instructed patients to avoid simultaneous ingestion of doxycycline and cation-containing preparations and excluded patients with kidney dysfunction.

The absence of a dose-response relationship in our study presumably indicates that the antiinflammatory effects in AAA are already maximal at the lowest dose of doxycycline used in this study (50 mg). Consistent with this, subantimicrobial doses of doxycycline (20 mg BID) are approved by the Food and Drug Administration for inhibition of excess MMP8 and MMP9 activity in periodontitis,[26] showing that low-dose systemic doxycycline treatment has clinical effects. For logistical reasons, the treatment period in this study was relatively short (2 weeks), yet observations from other chronic inflammatory conditions involving neutrophils such as periodontal disease, acne, and rosacae[7] suggest that the effects of doxycycline persist during prolonged treatment.

A molecular explanation for the reduction of the aortic wall neutrophil content after doxycycline treatment may be the reduction of neutrophil chemoattractants such as IL-8[27] in the aneurysm wall. This notion is supported by the robust correlation between aortic wall IL-8 levels and aortic wall neutrophil content that is present in the nontreated controls and that is lost after doxycycline treatment. The possible reduction of G-CSF levels ($P<0.00308$, NS) may contribute to this effect because G-CSF reportedly stimulates survival, differentiation, and function of neutrophil precursors and mature neutrophils.[28,29] Available preclinical studies suggest that doxycycline may also dose dependently interfere with neutrophil migration.[30] Yet, the relevance of this effect has been questioned because of the results of an earlier clinical study.[31]

The selective reduction of CD8$^+$ T cells, along with their activation markers perforin and granzyme A, after doxycycline treatment has not been reported previously. Our data demonstrate that the observed reduction of CD8$^+$ T cells is selective because there was no effect on CD4$^+$ T cells. To the best of our knowledge, T-cell trafficking and homing signals concur. In the absence of an effect of doxycycline treatment on CD4$^+$ T cells, we hypothesize that the reduction presumably reflects a direct effect on CD8$^+$ T cells. There is evidence for diverging roles of specific signaling pathways in CD8$^+$ T-cell and CD4$^+$ T-cell biology. For example, specific roles for the AP-1[32] and IL-6/STAT3[33] signaling pathways have been reported in the context of cytotoxic T-cell proliferation (AP-1) and survival (IL-6/STAT3). In the absence of known direct toxic effect of doxycycline on cytotoxic T cells,[17] we speculate that the selective reduction of cytotoxic T cells on doxycycline treatment may be mechanistically linked to the reduced AP-1 and/or STAT-3 activation after doxycycline therapy.

Direct inhibition of the AP-1 pathway has been shown to prevent and even reverse aneurysm formation in animal models of aneurysmal disease.[34] This published report showed not only that AP-1 is critically involved in the inflammatory cascade and the proteolytic imbalance in AAA but also that reduced AP-1 signaling resulted in increased matrix deposition.

We found that doxycycline therapy reduced aortic wall G-CSF levels. G-CSF expression is under primary control of C/EBP transcription factors. All 3 isoforms (α, β, δ) are elevated in AAA,[22] and increased aortic C/EBP expression is positively associated with aortic wall inflammation in atherosclerotic occlusive disease.[35] Antiinflammatory strategies that reduce C/EBP expression have been described to also reduce atherosclerotic disease.[36] Yet, little is known about the pathophysiological role of C/EBP in the context of AAA, and further mechanistic studies are necessary to investigate the contribution of C/EBP to AAA growth.

Conclusions

We report that a period of 2 weeks of doxycycline treatment in patients with advanced abdominal aneurysms results in a profound and selective reduction of aortic wall neutrophil and cytotoxic T-cell content. This cellular effect is paralleled by a selective suppression of inflammation on the molecular level, ie, cytokines (IL-6, IL-8) and transcription factors (AP-1, C/EBP, STAT3), which are relevant for neutrophil and cytotoxic T-cell inflammation, as well as by a specific reduction of neutrophil-derived proteases. Our findings show that doxycycline therapy may be a useful strategy to selectively quench specific cellular and molecular aspects of vascular inflammation in AAA. The ability of doxycycline to reduce aneurysmal growth awaits confirmation in a sufficiently powered clinical trial. These data provide a promising avenue for further research evaluating the efficacy of doxycycline in other chronic vascular and nonvascular inflammatory conditions involving neutrophils and/or cytotoxic T cells such as Kawasaki disease[37] and chronic obstructive pulmonary disease.[38]

Disclosures

None.

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CLINICAL PERSPECTIVE

Pharmaceutical stabilization of abdominal aneurysms, thereby reducing the need for aneurysm repair, holds many promises. Matrix metalloproteinase-9 is considered pivotal to the process of aneurysm formation. Because of its ability to reduce matrix metalloproteinase-9 expression and activity, the tetracycline analogue doxycycline has been brought forward as a promising lead candidate. Although animal studies have convincingly shown that doxycycline inhibits both aneurysm formation and growth, the effects of doxycycline on matrix metalloproteinase-9 are controversial in human studies. In this study, we confirm that doxycycline lowers aneurysmal wall matrix metalloproteinase-9 protein levels but also show that this reduction may be secondary and related to an effect on neutrophils, a cell type loaded with preformed matrix metalloproteinase-9 protein. We demonstrate that doxycycline has a profound but selective suppressive effect on inflammation in abdominal aortic aneurysms; doxycycline specifically lowers aortic wall neutrophil (−76%) and cytotoxic T-cell (−96%) content, 2 types considered crucial for aneurysm formation. Moreover, it is shown that treatment reduces interleukin-6 and -8 hyperexpression, a key feature of abdominal aortic aneurysm. Results of this study are relevant for pharmaceutical stabilization of the abdominal aneurysm and possibly for other (vascular) inflammatory conditions involving neutrophils and/or cytotoxic T cells such as Kawasaki disease and Behçet syndrome.