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Doxycycline Treatment Reduces Ischemic Brain Damage in Transient Middle Cerebral Artery Occlusion in the Rat

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Abstract

Agents that inhibit leukocyte adhesion including intercellular adhesion molecule-1 antibodies (anti-ICAM-1) have shown beneficial effects in experimental central nervous system (CNS) ischemia. Doxycycline inhibits leukocyte function in vitro by binding divalent cations and reduces spinal cord reperfusion injury. The authors used a clinically relevant model of focal CNS reperfusion injury to test whether treatment with doxycycline would reduce cerebral ischemic damage and improve functional outcome. Reversible middle cerebral artery occlusion was produced in adult Sprague-Dawley rats by advancing a filament into the internal carotid artery for 2 h. Animals received either IP doxycycline (10 mg/kg) (N = 13) or saline (N = 11) 30 min before ischemia, followed by 10 mg/kg every 8 h × 6. Both functional assessment (5 point neurologic scale) and infarct volume was evaluated at 48 h. Functional efficacy: doxycycline 0.5 ± 0.2 (mean ±SE) vs control 1.3 ± 0.3 (p = 0.03). Infarct volume: doxycycline 56 ± 18 mm^3 vs control 158 ± 44 mm^3 (p = 0.03); This protective effect supports the role of doxycycline in reducing CNS reperfusion injury.

Index Entries: Cerebral ischemia; doxycycline; therapy; leukocyte adhesion.

Introduction

Leukocyte appearance in central nervous system (CNS) ischemic tissue has previously been considered to represent a pathophysiological response to existing injury. Recent evidence suggests that leukocytes may also be directly involved in the pathogenesis and extension of CNS ischemic injury (Engler et al., 1986; Schmid-Schonbein and Engler, 1986). Two proposed mechanisms of leukocyte involvement in ischemia are: Direct microvascular occlusion from endothelial and basement membrane adhesion (Mori et al., 1987; Schmid-Schonbein, 1987); and transendothelial migration with secondary CNS tissue infiltration and neuronal cytotoxic injury (Schmid-Schonbein and Engler, 1986). Initial adhesion of leukocytes to microvascular endothelium is essential for either of these mechanisms.

Leukocyte adhesion to the endothelium is primarily mediated by the leukocyte adhesion molecule (CD 18) and its endothelial ligand, the intercellular adhesion molecule (ICAM-1) (Smith et al., 1988; Argenbright et al., 1991). The role of leukocyte

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adhesion and infiltration in potentiating CNS ischemia is supported by experimental studies that have reduced CNS ischemic injury by blocking leukocyte adhesion with anti ICAM-1 or CD-18 antibodies (Chopp et al., 1987; Clark et al., 1991; Zhang et al., 1994). Doxycycline, a tetracycline antibiotic that inhibits leukocyte adhesion by binding divalent cations, which are required for ICAM-1 to CD-18 receptor interaction (Gabler and Creamer, 1991), has been shown to improve neurologic function in experimental spinal cord ischemia (Clark et al., 1994), and inhibit leukocyte infiltration in experimental forebrain ischemia (Clark et al., 1995a). The present study evaluates the effectiveness of doxycycline in reducing neurologic damage and improving functional outcome in a clinically relevant reperfusion stroke model.

Materials and Methods

Ischemic Animal Model

All procedures were approved by the OHSU animal care committee. Focal cerebral ischemia was produced using an intraluminal vascular occlusion model (Zea Longa et al., 1989). Briefly, adult male Sprague-Dawley rats weighing 250–350 g were intubated under halothane/O_2 anesthesia, and respiration was mechanically controlled using a Harvard rodent ventilator. The tidal volume (2–2.5 mL) and rate (80 ± 2/min) were adjusted to keep arterial pCO_2 at 30–40 torr. The ventral tail artery was catheterized for monitoring mean arterial pressure (MABP), and to obtain blood samples. The rectal temperature was controlled at 37 ± 0.5°C with a feedback-regulated heating pad. The right common carotid artery, external carotid artery (ECA), and internal carotid artery (ICA), were isolated via a longitudinal median neck incision under an operating microscope. Approximately 19.0 mm of 4-0 surgical nylon suture was advanced from the lumen of the ECA into the ICA, to block the origin of the middle cerebral artery (MCA). Anesthesia was discontinued, the animals were extubated, and supplemental O_2 was administered until the respiratory efforts were normal. After 2 h of MCA occlusion, the animals were reanesthetized with halothane, and restoration of MCA blood flow was accomplished by withdrawing the intraluminal suture until the tip cleared the ICA lumen, and was retained in the stump of the ECA. Arterial blood pressure was monitored during MCA occlusion and arterial blood was sampled for serial measurements of pH, pO_2, glucose, and pCO_2.

Experimental Design

Animals were randomly divided into two groups. The doxycycline group ($N = 13$) received 10 mg/kg IP of doxycycline (Sigma, St. Louis, MO) 30 min prior to ischemia followed by 10 mg/kg IP every 8 h for 48 h. This dose of doxycycline has been shown to be neuroprotective in rabbits (Clark et al., 1994), and reduces hepatic ischemia in rats (Smith and Gabler, 1994). A saline-treated ischemic group ($N = 11$) received an equal volume of normal saline 30 min prior then every 8 h IP for 48 h.

Animals were weighed prior to ischemia and at 24 and 48 h after onset of ischemia. At 48 h, all animals were scored blindly on a six point scale: 0= normal; 1= drags forepaw, twisting when lifted; 2= circling spontaneously; 3= falls; 4= does not walk, comatose; 5= dead (Zea Longa et al., 1989).

Tissue Preparation and Infarct Volume

At 48 h postischemia, animals were anesthetized and tissues were fixed via intracardiac perfusion of 10 mL sodium phosphate buffer (100 m*M*, pH = 7.2) followed by 300 mL of 4% formaldehyde in phosphate buffer. To confirm that the inserted filament passed the origin of the MCA, animals were injected iv with 1 mL of 2% Evans blue dye prior to perfusion (Zhang et al., 1994). Brains were removed and immersion fixed with 4% formaldehyde in phosphate buffer for 48 h at room temperature. The tissue was equilibrated with a 15% sucrose solution containing 0.05% sodium azide, followed by a 30% sucrose solution containing 0.05% sodium azide. The tissue was cut into serial 50-μ thick sections on a freezing sled microtome.

Ten 50-μ coronal sections from interaural +13.7 to interaural +1.7 were selected at 1.2 mm intervals from each brain, and were stained with Luxol Fast Blue and Cresyl Violet. The infarcted area and the ipsilateral hemispheric area (in square millimeters) were measured (traced) using an Image ProPlus analysis program. The volumes (in cubic

Table 1
Physiological Variables[a]

	pO_2 (mm Hg)		pCO_2 (mm Hg)		pH		Glucose (mg/dL)		MABP	
Group	Pre	Ischemia	Pre	Ischemia	Pre	Ischemia	Pre	Ischemia	Pre	Ischemia
Control	130 ± 17	148 ± 15	29 ± 6	38 ± 4	7.5 ± 0.1	7.4 ± 0.0	83 ± 14	74 ± 17	–	97 ± 08
Doxy	121 ± 21	144 ± 13	31 ± 8	37 ± 3	7.5 ± 0.1	7.4 ± 0.1	77 ± 12	65 ± 10	–	95 ± 08

[a]Values are mean ±SD. Measurements preischemia were made 10–15 min before MCA occlusion. Measurements of ischemia were made 10–15 min after occlusion.

Table 2
Animal Weights and Peripheral Leukocyte Counts Before and After MCA Occlusion

Group	Time	WBC × 1000/mm^3	Weight, gs
Control (N = 11)	Before	9.5 ± 1.8	292 ± 09
	24 h	—	275 ± 12[a]
	48 h	7.0 ± 3.8	273 ± 18[a]
Doxy (N = 13)	Before	10.4 ± 3.0	294 ± 25
	24 h	—	283 ± 16
	48 h	5.7 ± 2.5[a]	282 ± 17

[a]$p < 0.05$ compared to baseline.

millimeters) were determined by multiplying the appropriate area by the section interval thickness. To avoid errors associated with processing the tissue for histological analysis, the infarct volume size was also presented as the percentage of lesion to the ipsilateral hemisphere.

Immunohistochemistry

Immunohistochemical staining was used to determine if doxycycline treatment was associated with a reduction in leukocyte infiltration at 48 h. Sections were incubated overnight with: ED1 (mouse monoclonal antirat monocyte/macrophage 1:000 dilution. Serotec, Oxford, UK), HIS48 (mouse monoclonal antirat granulocyte. 1:50 dilution. Serotec) or normal mouse serum (1:2000 dilution), and then developed using diaminobenzidine as a substrate. The mean cell density in a section through the middle of the infarct (section with largest infarct area) was determined by averaging the number of cells present in three representative 1 mm^2 areas in the parietal cortex, basal ganglia, and preoptic cortex. Two sample t-tests, with Bonferroni's correction, were performed to detect differences between groups ($p < 0.5$).

Results

A total of 24 animals were studied. Evans blue staining confirmed the successful placement of the suture past the MCA in all animals. The values for arterial blood pressure, pO_2, pCO_2, serum glucose, and pH before and during MCA occlusion are summarized in Table 1, and are not significantly different among the groups. Rectal temperature was controlled at 37 ± 0.5°C in all animals. The mean ±SD animal weights before and at 24 and 48 h after MCA occlusion are shown in Table 2. Within the control group, a significant decline of weight was detected at 24 and 48 h compared with baseline values ($p < 0.5$). The weights in the doxycycline treated animals did not significantly decline from their baseline values. The total leukocyte counts for each group are also shown in Table 2. Compared with baseline values, both groups had reductions in postischemic baseline leukocyte counts.

The results of neuroprotective assessments are shown in Table 3. Treatment with doxycycline significantly improved the clinical functional assessment at 48 h: 0.5 ± 0.2 (mean ±SE) vs control 1.3 ± 0.3 ($p = 0.03$). Treatment with doxycycline also reduced both the absolute infarct volume: 56 ± 18 mm^3 vs

Table 3
Clinical Score, Infarct Volumes, and Percent Lesion Volume to the Ipsilateral Hemisphere in the Four MCA Groups

Group	Score (0–5)	Lesion, mm^3	% Lesion V.
Control ($N = 11$)	1.3 ± 0.3	158 ± 44	21.6 ± 5.8
Doxy ($N = 13$)	0.5 ± 0.2[a]	56 ± 18[a]	8.6 ± 2.7[a]

[a] $p < .05$ compared to control. Values are mean ±SE.

control 158 ± 44 mm^3 ($p = 0.03$); and the percent volume of the lesion to the ipsilateral hemisphere 8.5 ± 2.7% vs 21.7 ± 5.8% ($p = 0.04$).

To determine if doxycycline reduced leukocyte infiltration, immunohistochemistry was done at 48 h. Staining revealed only rare (< 1 cell/mm^2) His48-positive cells (PMNs) in both doxycycline and control groups. Staining with ED1 (monocyte/macrophage) revealed an average of 232 ± 69 cells/mm^2 in infarcted tissue from control animals, compared to 227 ± 57 cells/mm^2 in doxycycline treated animals (NS). The ramified morphology of these ED1 positive cells appeared to be similar to that of microglia cells.

Discussion

This study found that administration of the antileukocyte adhesion therapy doxycycline significantly reduced ischemic injury, and improved functional outcome in a focal CNS reperfusion model. Doxycycline treatment did not affect the accumulation of macrophages at 48 h as assessed by immunohistochemistry.

The MCA occlusion model used in this study closely approximates the most common type of clinical stroke with associated vessel recanalization (thrombolysis). The magnitude of the neuroprotection seen with doxycycline treatment in this study is similar to previous studies using antileukocyte adhesion agents using the same MCA reperfusion model (Chopp et al., 1991; Matsuo et al., 1994; Zhang et al., 1994). Doxycycline, a member of the tetracycline family of antibiotics, inhibits in vitro human leukocyte superoxide synthesis, degranulation, and adherence to protein-coated surfaces (Gabler and Creamer, 1991; Gabler et al., 1992). These inhibitory effects on leukocyte function are felt to be produced by Dc binding of the divalent cations Mg^{2+} and Ca^{2+} (Gabler and Tsukuda, 1991), which are required for the CD18 to ICAM-1 interaction. The authors previously have found doxycycline to be neuroprotective in an experimental spinal cord ischemia model when treatments were given either 30 min pre or 5 min post the onset of ischemia (Clark et al., 1994). In that study, Dc treatment also significantly inhibited both neutrophil and mononuclear cell concurrent in vitro adhesion (Clark et al., 1993). These beneficial effects of Dc treatment in reperfusion injury may seem paradoxical in that an antibiotic is blocking leukocyte function. The explanation is that although all tetracyclines appear to have anti-inflammatory properties, they have a far greater ability to inhibit bacterial replication (Gabler and Creamer, 1991).

The authors previously have demonstrated that doxycycline is sequestered in the CNS (Clark et al., 1994). Because doxycycline binds Ca^{2+} and Mg^{2+}, an alternative explanation for the observed neuroprotective effects is that doxycycline is decreasing the locally available Ca^{2+} in the ischemic CNS tissue. This could directly inhibit the excitatory ischemic cascade that is felt to be detrimental to CNS tissue during ischemia (Rothman and Olney, 1986). Further work is needed to confirm the exact neuroprotective mechanism.

The authors previously have found significant infiltration of PMNs in transient ischemia using HIS48 immunohistochemistry at 24 h, but not at later time points (Clark et al., 1995b), and that doxycycline treatment inhibited the PMN infiltration at 24 h (Clark et al., 1995a). In the current study, the authors were unable to detect significant numbers of PMNs in either doxycycline-treated or control animals at 48 h. Consequently, the authors were unable to assess whether doxycycline treatment inhibited PMN infiltration. They were also unable to detect any doxycycline treat-

ment effects on macrophage accumulation. The monoclonal antibody used, ED1, has been shown to recognize a cytoplasmic antigen in rat monocytes and macrophages (Dijkstra et al., 1985). ED1 is not expressed by nonactivated microglia cells in normal rat brain (Graeber et al., 1989). The authors previously have found that treatment with doxycycline inhibited monocyte/ macrophage infiltration at 24 h, but not at later time points (Clark et al., 1995a). The ramified morphology of the ED1 positive cells in the current study along with the failure of doxycycline treatment to reduce the accumulation suggest that these cells represent transformed resident microglia. It is possible that doxycycline would have reduced both ED1 and HIS48-positive cellular infiltration if the brains had been examined at 24 h.

Based on the encouraging results with anti-ICAM-1 therapies in experimental models of CNS reperfusion, a randomized multicenter clinical stroke treatment trial using a murine anti-ICAM-1 was recently completed ("Enlimomab for Treatment of Acute Ischemic Stroke," Boehringer Ingelheim Pharmaceuticals, Inc.). Surprisingly, anti-ICAM-1 treatment increased mortality and worsened functional outcome (Sherman, 1997). Some of the proposed reasons for this adverse treatment effect include an immune reaction to the mouse MAb used or complement binding to the large anti-ICAM-1 antibody itself. By avoiding these immune effects, doxycycline may prove to be a safe, inexpensive, and readily available antiadhesion therapeutic alternative. Finding an agent that could safely reduce CNS reperfusion injury would have widespread clinical benefit following thrombolysis, or as a pretreatment in clinical situations where the risk of stroke is high.

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