ONLINE DATA SUPPLEMENT

The Inflammatory Pre-atherosclerotic Vascular Remodeling Induced by Intermittent Hypoxia is Attenuated by RANTES/CCL5 Inhibition

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METHODS

Animals

Eight weeks old male C57BL/6J mice (CERJ Janvier) were used. All the experiments were conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Council of Europe, European Treaties ETS 123, Strasbourg, 18 March 1986), and with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996). Mice were randomized to 5 or 14 days exposure to intermittent hypoxia (IH) or air (normoxia: N). All efforts were made to reduce the number of animals used to an unavoidable minimum.

Intermittent hypoxia

IH was performed as previously described (E1). The animals were exposed to 8h of IH or N (from 7-8 am), and regularly weighed during the exposure period (Figure EA). FiO_2 in the hypoxic chambers was measured with a gas analyzer (ML206, ADInstruments) throughout the experiment. The IH stimulus consisted of a 60s cycle, with 30s hypoxia (hypoxic plateau at 5.% FiO_2) and 30s normoxia (normoxic plateau at 21% FiO_2). Normoxic mice were

exposed to air stimulus at similar flows than IH stimulus thereby simulating similar levels of noise and turbulences related to gas circulation. Ambient temperature was maintained at 20-22°C. The day following the last exposure period, animals were anesthetized under ketamine-xylazine; blood was collected from cardiac puncture for hematocrit, chemokine and total cholesterol determinations, and tissues were harvested and immediately frozen in liquid nitrogen, then stored at -80°C until analysis.

Hematocrit and Cholesterol determinations

The hematocrit was measured in microcapillary tubes and plasma total cholesterol was measured using enzymatic technique (Modular, Roche Diagnostics).

Assessment of systemic inflammation

Systemic inflammation was assessed through the cellular inflammatory response which is known to be involved in the early steps of atherogenesis (E2). Three indices of activation of the inflammatory cells were assessed on total spleen-derived cells (splenocytes) that include mainly lymphocytes with predominant T-cells, and some monocytes and neutrophils.

Proliferation Assay

Splenocytes were isolated from mice exposed to IH or N for 5 or 14 days (n=6-12 per group), and cultured in 96-well plates at a concentration of $5x10^6$ cells.ml-1. Culture medium consisted of RPMI 1640 supplemented with 25 mM HEPES buffer, 2 mM L-glutamine, 100 U.ml-1 penicillin, 0.1 mg.ml-1 streptomycin and 10% heat-inactivated FBS. Cells were stimulated in triplicates with graded concentrations of the mitogenic factor concanavalin-A (Sigma). Forty-eight hours later, cell proliferation was determined using a non-radioactive MTS cell proliferation assay (Promega).

Splenocyte Chemokine mRNA Expression

Chemokine expression was assessed by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Total mRNA was extracted from splenic tissue of hypoxic and normoxic mice exposed for 5 or 14 days (n=6 per group), isolated with Tri-reagent (MRC, Inc) and further analyzed by real-time qRT-PCR (ABI Prism 7000 Sequence Detection system, Applied Bio-systems). MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4 and RANTES/CCL5 mRNA expression was determined as previously described (E3). Each sample was analyzed in triplicate and normalized to the expression levels of the eukaryotic 18S ribosomal-RNA as an endogenous control unaffected by hypoxia (TaqMan Reagent, Applied Biosystems; VIC labelled). The analysis was performed with ABI Prism software, and the fold induction in mRNA levels was calculated using comparative Ct method (Applied Biosystems).

Ex vivo transmigration assay

Splenocytes were isolated from mice exposed to 14 days of IH or N, and stimulated (n=4, in duplicate) for 4h with interferon-gamma (50 ng.ml-1). Stimulated and unstimulated cells were transferred into the upper compartment of transwell filter migration chambers. Migration was triggered by the addition of RANTES/CCL5 (10 ng.ml-1, R&D Systems) to the lower transwell chamber compartment. After 60 min of incubation, the number of migrated cells was determined by counting 10 microscopic fields per well with two blinded independent observers.

Assessment of vascular inflammation

Leukocyte-endothelium interactions were assessed in the mesenteric microcirculation whereas structural remodeling was evaluated in descending thoracic aortas. Adhesive interactions between leukocyte and microvessels were studied by leukocyte rolling and ICAM-1 adhesion molecule expression. Indeed, leukocyte recruitment into inflamed tissues involves different steps including successively leukocyte rolling, adhesion and transmigration through the endothelium. The rolling corresponds to repeated contacts of leukocytes with the endothelial cells. Leukocyte arrest and adhesion on the endothelium surface is due to the interaction between the activated leukocytes and endothelium ligands such as the adhesion molecule ICAM-1. Leukocyte rolling was measured with intravital microscopy as previously described, and required thin transparent tissue such as mesentery. Since early alterations could occur in the course of IH exposure, different time points were assessed, with day 5 as the earliest. In contrast, structural changes were investigated after 14 days of exposure and concerned the thoracic aorta, since its structure allows the analysis of the different tunica like in the human arteries.

1. Mesenteric resistance arteries

Leukocyte rolling

Leukocyte rolling was analyzed using intravital microscopy on exteriorized mesenteric microvasculature from mice exposed to IH or N during 5 or 14 days (n=4-5 per group). Leukocytes were stained by injecting the mice intravenously with 50 µl of 0.05% rhodamine 6G (Sigma), 5 min before microscopy. Leukocyte rolling was then observed microscopically, as previously described (E4). The number of rolling leukocytes was determined by two blinded independent observers, in five 20-40 µm diameter post-capillary venules for each

animal, as the number of rolling cells moving past a fixed point on the venular wall per 30 second period of time.

ICAM-1 protein expression

Expression of ICAM-1 protein was assessed by Western blotting of mesenteric lysates from mice exposed to 14 days of IH or N (n=4 per group). Mesenteries were harvested, homogenized in RIPA buffer (Tris HCl 50 mM, NaCl 150 mM, NP-40 1%, sodium deoxycholate 0.5% and protease inhibitors) and centrifugated at 13 000 rpm for 20 min to remove nuclei and debris. Supernatants were collected and protein concentrations were determined (Pierce assay, Perbio Science). Proteins (70 µg per sample) were separated on 8% SDS-PAGE, electrotransferred to nitrocellulose membrane, and immunoblotted for ICAM-1 using a rabbit polyclonal antibody (Santa-Cruz Biotechnology). Equal protein loading was verified by Ponceau staining of the membranes. Relative densitometry was assessed using Image J® software.

2. Thoracic aorta

Immunohistochemistry

IH and N aortas were embedded in OCT compounds (Tissue-Tek, Sakura), sectioned (10 µm) and stained. Hematoxylin-eosin staining was used to assess global tissue morphology. Verhoeff, alcian blue, Oil-red-O and sirius red stainings were performed to assess elastic fibers (fiber thickness and distance between fibers), mucoid degeneration, lipid deposition and collagen content of the aortic wall, respectively.

For immunostainings, sections were fixed in acetone (CD3 staining) or dried without fixation (RANTES/CCL5 staining). Endogenous peroxidase activity was inactivated with 1% (v/v) hydrogen peroxide and non-specific binding was blocked with 2% (w/v) non-fat dried milk

(CD3 staining) or 1-3% BSA (RANTES/CCL5 staining) in PBS, pH 7.4. Sections were incubated overnight at 4°C with a rabbit anti-human CD3 (1:1000, #A0452, DakoCytomation) or a goat anti-mouse RANTES/CCL5 antibody (1:40, #AF478, R&D systems). After washes, the primary antibody was detected either directly using the DakoCytomation EnVisionTM+HRP system, or after a 1h incubation with a biotinylated rabbit anti-goat antibody (1:300, #305-066-045, Jackson ImmunoResearch), followed by DAB staining and hematoxylin counterstaining. Control sections were prepared by substituting rabbit IgG (CD3 staining) or goat Ig (2 μg/ml, RANTES/CCL5 staining) for primary antibodies.

Morphometric analysis was performed with a light microscope (Eclipse 80i, NIKON France S.A.) and LUCIA-G software Version 5.0 (Laboratory Imaging Ltd, Prague, Czech Republic). It included measurements of IMT and surface, aorta internal perimeter, fiber thickness and distance separating the elastic lamellae, nuclei counting of SMC of the tunica media, and counting of CD3-positive cells in the different layers of the aortic wall. For each animal (n=6-10 mice per group), a mean value of each parameter was obtained by averaging 8-10 different measurements spanning the entire cross-section of 2-3 non-contiguous sections at 10 x 10 or at 10 x 40 magnification.

Nuclear NFkB-p50 expression

As activation of the proinflammatory transcription factor NFkB requires nuclear translocation (E5), we investigated whether IH could activate NFkB by assessing the nuclear expression of the NFkB-p50 subunit in the aortic tissue. Nuclear NFkB-p50 expression was determined in IH and N aorta (n=4 per group) that were homogenized in a lysis buffer containing 10 mM HEPES-KOH, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF and protease inhibitors (Complete, Roche Diagnostics) and centrifuged (10 min , 2900 rpm). The

supernatants (cytosolic fractions) and the pellets were resuspended in a buffer containing 10 mM HEPES-KOH, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF and protease inhibitors (Complete, Roche Diagnostics). The pellets were centrifuged 2 min at 12000 rpm, and the supernatants corresponding to nuclear fractions were collected. Proteins concentrations were determined using the Bradford assay (Biorad). Nuclear proteins (50 µg/lane) were separated on 10% SDS/PAGE, electrotransferred to nitrocellulose membrane and immunoblotted for p50-NFkB using a rabbit polyclonal antibody (Abcam). Proteins were visualized using enhanced chemiluminescence system (Pierce-Perbio). Equal loading was verified by Ponceau staining of the membranes. Relative densitometry was assessed with Image J® software.

ICAM-1 and alpha-smooth muscle actin protein expression

ICAM-1 and alpha-smooth muscle actin (αSMA) protein expression from IH and N aortas (n=4 per group) was assessed by Western blot, as described above. Tissues were homogenized and proteins were separated on 8% SDS-PAGE, immunoblotted for ICAM-1 (R&D systems) or αSMA (Abcam), visualized and quantified as described above. Equal protein loading was verified using GAPDH immunoblotting.

Aortic IFNγ mRNA expression.

Total mRNA was extracted from IH and N aortas, and IFNγ expression was assessed by quantitative RT-PCR, and normalized using ubiquitin mRNA levels as the internal control.

RANTES/CCL5 neutralization

To confirm the role of RANTES/CCL5 in IH-induced inflammatory vascular remodeling, we used a rat anti-mouse RANTES/CCL5 monoclonal antibody. The antibody selectively binds

to the chemokine RANTES/CCL5 and inhibits the interaction of RANTES/CCL5 with its receptors thereby neutralizing RANTES/CCL5 activity. Four additional groups of mice were therefore studied. Thirty male adult C57BL/6J mice were treated with either a rat anti-mouse RANTES/CCL5 monoclonal antibody (MAB478, R&D systems) or its rat IgG isotype control, and were exposed to either IH or N for 14 days. The IgG2a isotype control was purified from hybridoma clone mAb64 (ATCC). Antibodies were injected intraperitoneally, 3 times a week at a dose of 250 μg per mouse, during the 2 weeks of exposure. The dose and injection regimen (every 2 days) have been demonstrated to significantly reduce inflammatory processes in mice, with an effect lasting until 2 days after the injection (E6). Mice were sacrificed under anesthesia and descending aortas were collected for histomorphometry (intima-media thickness), immunostainings for CD3 and RANTES/CCL5, quantification of the expression of αSMA, nuclear NFkB-p50 and IFNγ mRNA, as described above.

RESULTS

As previously described (E7), the weight of IH-mice initially decreased then increased, paralleling weight evolution of normoxic mice (online Figure E1A). IH-mice had elevated hematocrit and plasma total cholesterol after 14 days of exposure (online Figures E1C and E1D). Alterations in body weight, hematocrit and total cholesterol persisted in IH-mice treated with the anti-RANTES/CCL5 antibody (online Figures E1B to E1D).

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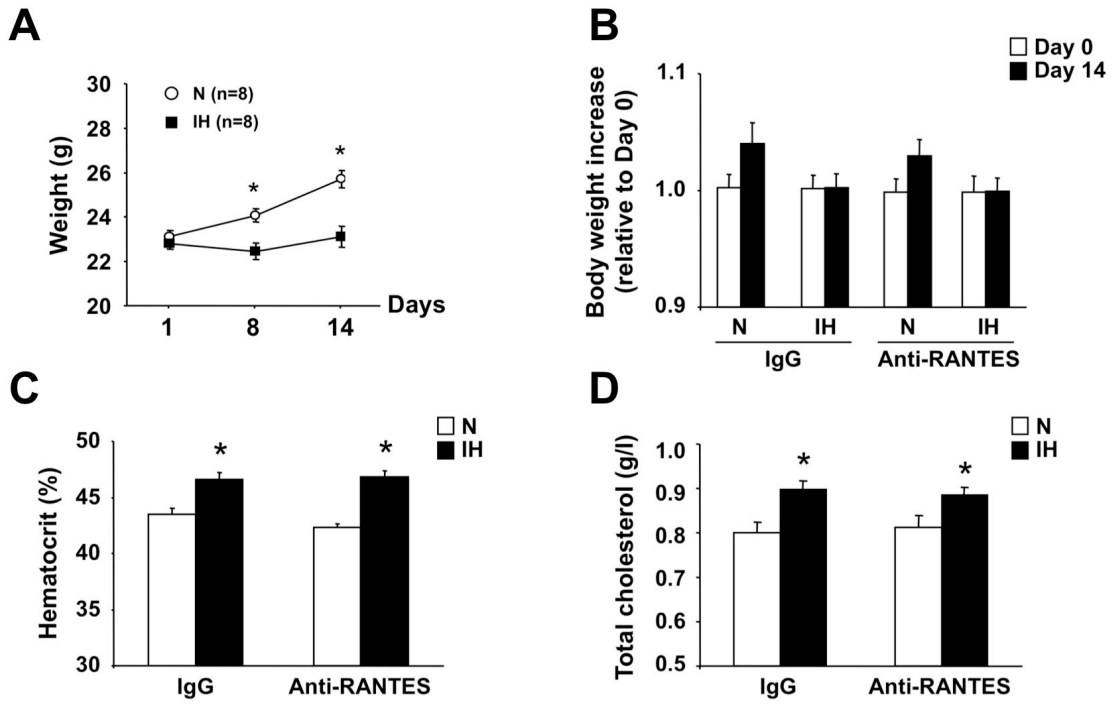
FIGURE LEGENDS

Figure E1. Body weight, hematocrit and cholesterol alterations induced by intermittent hypoxia

(A) Body weight evolution during a 14 day exposure to intermittent hypoxia (IH) or air (N). Body weight gain (B), hematocrit (C) and plasma total cholesterol (D) after 14 days of IH or N in mice treated with the monoclonal anti-RANTES/CCL5 antibody or the control IgG, *$p<0.05$ *vs* N, n=12 per group.

Figure E1


A
N (n=8)
IH (n=8)
Weight (g)
30
28
26
24
22
20
1
8
14
Days
*
*
B
Day 0
Day 14
Body weight increase
(relative to Day 0)
1.1
1.0
0.9
N
IH
N
IH
IgG
Anti-RANTES
C
N
IH
Hematocrit (%)
50
45
40
35
30
*
*
IgG
Anti-RANTES
D
N
IH
Total cholesterol (g/l)
1.0
0.9
0.8
0.7
0.6
0.5
*
*
IgG
Anti-RANTES