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Spontaneous Aortitis in the Balb/c Mouse

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Abstract

We examined whether high incidence rates (18%-56%) of inflammation in the root of the aorta detected in a Balb/c mouse model for hind limb ischemia were related to the surgical procedure. Twenty mice underwent ligation of the femoral artery; incidences of aortic root inflammation were compared to those observed in controls. We used a multiple-section sampling method to increase the sensitivity of the diagnostic rates. Although a cumulative incidence of 12.5% was found, no difference was seen in the overall incidence rates between the control and the surgically treated groups. Evaluation of blood levels of inflammatory cytokines showed that ligation of the femoral artery produced higher levels of interleukin-6 in the surgically transected group of mice. The development of spontaneous arteritis in this strain must be considered in future studies.

Keywords: limb ischemia; inflammation; aortitis; Balb/c.

Introduction

Vasculitis in animal models is induced experimentally and occurs spontaneously (Luzina and Handwerger 2000). To evaluate the potential for vascular toxicity in drug safety studies, investigators must know background ranges. Vasculitis was not reported in the past to occur spontaneously in the Balb/c mouse strain, except for one report of vasculitis-like lesions in male reproductive tissues, without involvement of other organs (Itoh et al. 1999; Luzina and Handwerger 2000). On the contrary, the Balb/c mouse strain was reported to be relatively resistant to the induction of vasculitis by *Candida albicans* extract (Takahashi et al. 2004). Nevertheless, the Balb/c strain has been used as an animal model to induce vasculitis experimentally using different techniques (Luzina and Handwerger 2000), among them knock-out of the interleukin (IL)-1 receptor antagonist gene (Matsuki et al. 2005; Shepherd et al. 2004). When evaluating vascular lesions, the aortic base, because of its unique location, poses a diagnostic challenge. The trimming method for this anatomical part is not specifically mentioned in the routinely used trimming guide (Morawietz et al. 2004). We describe, in the Balb/c mouse strain, the spontaneous occurrence of arteritis at the root of the aorta, initially attributed to femoral artery ligation, and the steps that were taken to identify these lesions correctly.

Animal handling in all of the experiments described in the current article followed guidelines of the National Institutes of Health and the Association for Assessment and Accreditation of Laboratory Animal Care. The study was approved by The Israel Board for Animal Experiments and was in compliance with The Israel Animal Welfare Act.

Experiment 1

Forty-three physically healthy, eight- to ten-week-old male Balb/c mice (approximate body weight 25 g) underwent femoral artery ligation as a model for hind limb ischemia. Surgically treated mice were used in a twenty-nine-day therapeutic and safety evaluation study of placental-derived mesenchymal stromal (PLX-PAD) cells. Mice were operated on and managed thereafter at Pharmaseed Ltd., Ness Ziona, Israel. The mice were examined at least twice daily for clinical status.

On day 1, under anesthesia (1%–1.5% isoflurane, 60% dinitrous monoxide, 40% oxygen), a 1- to 1.5-cm incision was made in the inguinal skin of all mice; the femoral artery was ligated twice and transected between the ligatures. Three treatment groups were used as follows: Group 1, vehicle control receiving PlasmaLyte (Baxter Healthcare Corp., Deerfield, IL) containing 10% dimethyl sulfoxide and 5% albumin once intramuscularly; Group 2, single intramuscular (IM) injection of PLX-PAD cells in PlasmaLyte; Group 3, two IM injections of PLX-PAD cells in PlasmaLyte. Five hours post-surgery, mice received an IM injection of vehicle (Group 1) or PLX-

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Abbreviations: IFN-γ, interferon-γ; IL, interleukin; IM, intramuscular; TNF-α, tumor necrosis factor-α.

TABLE 1.—Microscopic findings in the heart in male Balb/c mice in the first study.

Organ/tissue	Histopathological findings: no. of animals affected (% of incidence)		
	Group 1: vehicle control[a]	Group 2: 1×10^6 PLX-PAD (test product), single IM injection	Group 3: 1×10^6 PLX-PAD (test product), 2 IM injections
No. examined	11	23	9
Thrombosis at root of aorta	0 (0.0)	1 (4.3)	0 (0.0)
Myocardial fibrosis	1 (9.1)	0 (0.0)	2 (22.2)
Arteritis at root of aorta	2 (18.2)	6 (26.1)	5 (55.6)

[a] PlasmaLyte containing 10% dimethyl sulfoxide and 5% albumin.

PAD cells (Group 2), and Group 3 received a second injection on day 8. On day 29, animals were sacrificed, and organs were examined grossly for any abnormalities. Brain, salivary gland (right), lungs, thymus, spleen, testes (right), heart, liver, kidneys, lymph nodes (cervical, mandibular), trachea and thyroid (parathyroid), bladder, esophagus, pancreas, stomach, duodenum, jejunum, ileum, cecum, colon, sternum, and the quadriceps muscles of the ischemic and control legs were collected and fixed in 10% buffered formalin. Only the lungs, heart, and injection site were examined histologically.

Microscopic changes were seen in the heart (Table 1); inflammatory vascular lesions at the root of the aorta were the major finding (incidence rate: 18%–56%). No additional vasculitis was noted in the other examined organs (lungs and injection site).

Induction of acute ischemia may trigger a major systemic inflammatory reaction, accompanied by release of a variety of inflammatory cytokines, including IL-1 (Tang et al. 2005). Therefore, we suspected that the surgical procedure used in Experiment 1 caused the relatively high incidence of vasculitis by inducing the release of inflammatory cytokines at the base of the aorta, which is especially exposed to blood-flow stress (Matsuki et al. 2005).

EXPERIMENT 2

To determine whether pro-inflammatory cytokines released by ligation of the femoral artery were responsible for the damage to the aorta and its major branches, we performed a second experiment under conditions similar to those used in the first experiment. Forty mice were divided into two groups of twenty; one group served as unoperated control, whereas the other group underwent ligation of the femoral artery. Four days post-surgery, blood for cytokine analysis was withdrawn from both groups. Following sacrifice on day 29, a detailed postmortem examination was conducted. Cardiac samples with the entire aorta, including aortic arch, mediastinal arteries, abdominal aorta from diaphragm to iliac bifurcation, as well as the left kidney, spleen, pancreas, mesenteric lymph nodes with surrounding fat tissue and mesenteric blood vessels, and

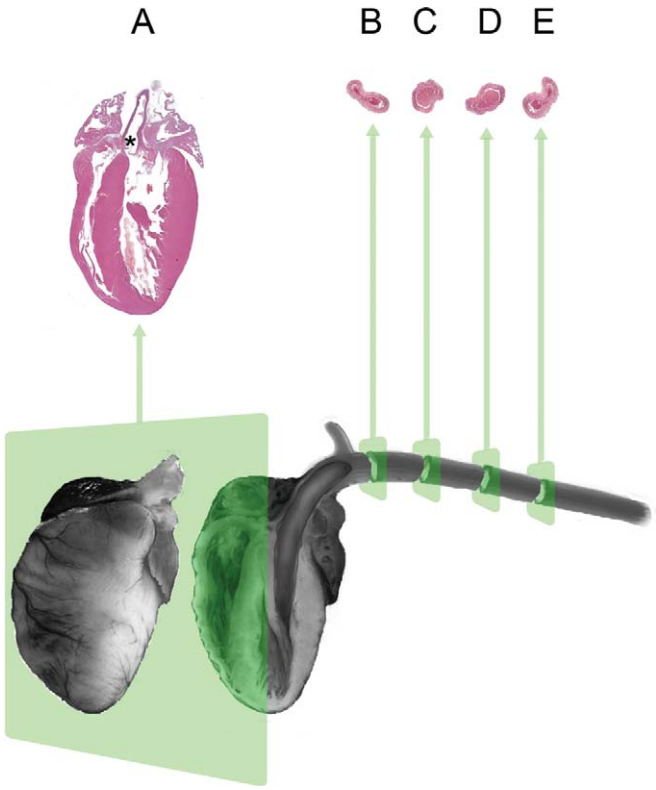

FIGURE 1.—Method of trimming the heart and aorta, showing the gross appearance of fixed tissues (lower) and the derived histological sections (upper): mid-longitudinal section of the heart (A), through both ventricles from base to apex, including 3 mm of aortic root and aortic arch (*); four sequential and transverse sections of the descending aorta, which are approximately 1 cm distant from each other (B-E). (Images of the heart are based on the NIEHS-NTP Heart Trimming Protocol).

TABLE 2.—Microscopic findings in the heart in male Balb/c mice in the second study.

Organ/tissue	Histopathological findings: no. of animals affected (mean severity[a])	
	Group 1: untreated	Group 2: hind limb ischemia
No. examined	20	20
Arteritis at root of aorta	3 (0.4)	2 (0.4)
Thrombosis at root of aorta	1	0

[a] Grading: 0 = no lesion, 1 = minimal change, 2 = mild change, 3 = moderate change, 4 = marked change.

quadriceps muscles of the ischemic and control legs with the leg arteries, were fixed in 10% buffered formalin.

Following the findings from Experiment 1, indicating that the pathology was localized to the aortic root, particular attention was paid to the histological preparation of the heart and descending portion of the aorta. Hearts, including the aortic arch and the remaining part of the descending aorta, were processed in their entirety. In this experiment, we used a

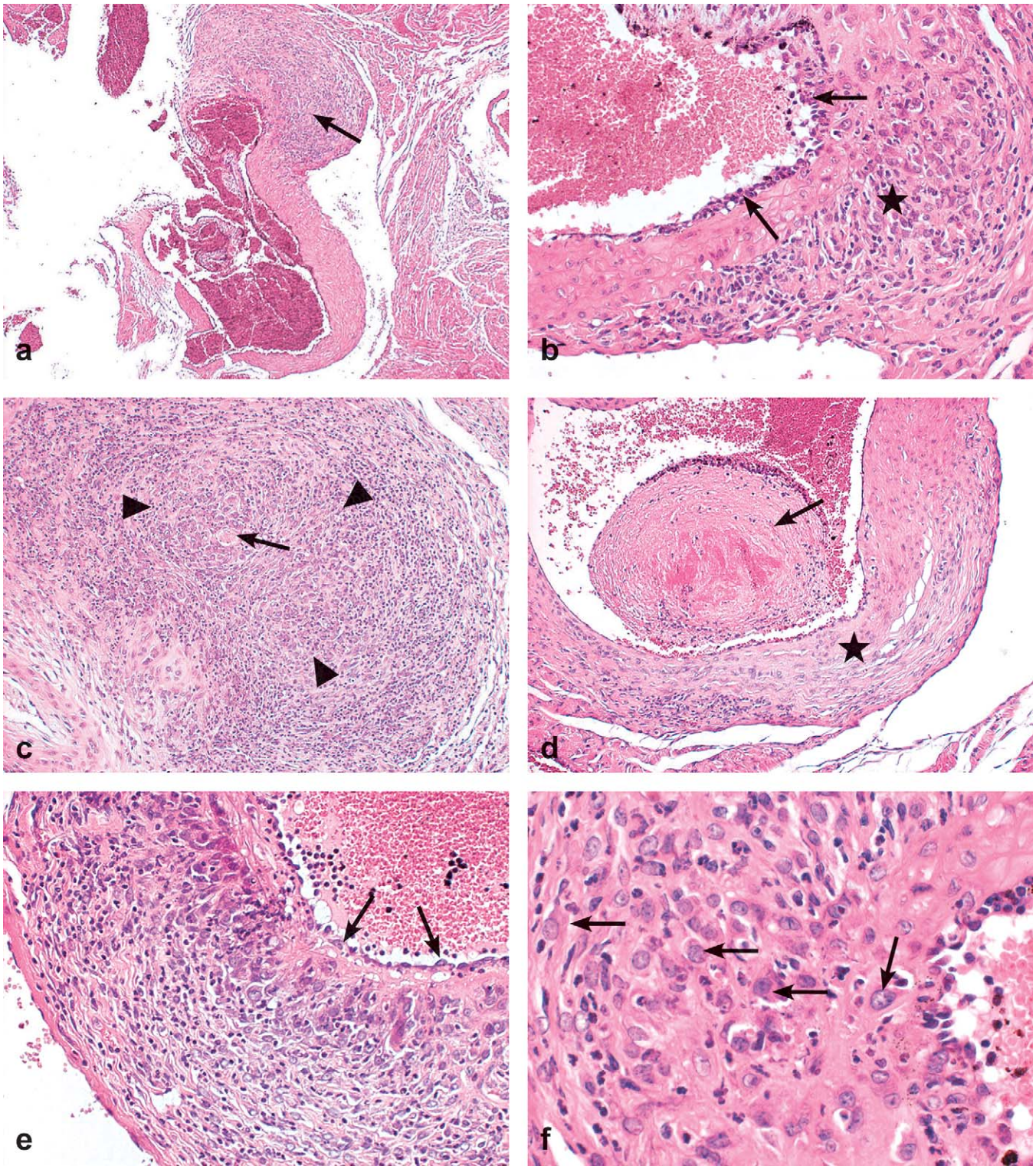

FIGURE 2.—(a) Focal thickening of aorta at its root. Arrow: mixed inflammatory reaction within wall. Hematoxylin and eosin (H&E), ×4. (b) Higher magnification of (a). Subendothelial polymorphonuclear and mononuclear cell infiltration (arrows) and mixed inflammatory cell infiltration within media and adventitia (asterisk). H&E, ×20. (c) Root of aorta. Arrowheads: inflammatory reaction occluding the aortic lumen. Arrow: possibly newly formed anastomizing (collateral) vessel, bypassing site of obstruction. H&E, ×10. (d) Thrombus (arrow) close to chronically inflamed aortic root (asterisk). H&E, ×33. (e) Aortitis at root. Transmural inflammation consists of numerous macrophages, polymorphonuclear cells, and lymphocytes. Endothelial lining exhibits cuboidal (''activated'') appearance (arrows). H&E, ×20. (f) Higher magnification of (e). Note numerous large macrophages (arrows) with intermixed polymorphonuclear cells. H&E, ×40.

TABLE 3.—Plasma levels of pro-inflammatory cytokines.

Cytokine level, pg/mL	Untreated animals	Hind limb ischemia animals	*p*	No. examined
IL-12	694.9 ± 201.3	868.2 ± 230.5	.131	8
IL-2	51 ± 70.3	16.4 ± 42	.252	8
IL-6	1099 ± 376.3	1833 ± 546*	.038	5
TNF-α	267.8 ± 36.8	295.6 ± 79.5	.549	4
IFN-γ	1244.1 ± 153.2	1261 ± 394.1	.886	3

Values are mean ± SD.
* $p < .05$ vs. normal mice (unpaired, two-tailed Student *t*-test).

"multiple-section sampling" method, which was not used in Experiment 1. We used this method to ensure that the aortic root, despite its relatively small size, would be present in the majority of heart sections examined. This method was previously proven to increase diagnostic rates in renal carcinogenicity studies (Eustis et al. 1994). In addition, it enabled us to determine more accurately the extent of the inflammatory lesions. The method of trimming of the heart and aorta is presented in Figure 1. A paraffin block was made that included the heart, together with the initial 3-mm portion of the aorta and the four sequential anatomical portions of the descending aorta; 5-μm sections were cut at a distance of approximately 30 to 40 μm, to obtain a total of four or five sections that were finally stained with hematoxylin and eosin (H&E). The aortic root was present in two to five sections per animal. The remaining collected tissues were embedded in three separate paraffin blocks, and 5-μm sections were cut and stained with H&E.

Microscopic changes at the root of the aorta were seen in three control mice and two that had undergone surgery (Table 2). The cumulative incidence of aortitis was five of forty mice (12.5%). The occurrence of these lesions in both surgically transected and control groups at comparable incidence indicated a spontaneous origin for the inflammation that was unrelated to surgical intervention. The morphological characteristics of aortitis were similar in both experiments. The changes were either focal (Figure 2a, 2b), leading to thickening of the arterial wall in a limited segment, or circumferential. In one animal from the surgically transected group, the lumen was almost completely obstructed (scored as grade 4), leaving only a small vessel (Figure 2c, arrow), likely representing a newly formed anastomizing (collateral) vessel, bypassing the site of obstruction. In an animal from the control group, a thrombus (scored as grade 2) occurred close to the site of the chronically inflamed wall (Figure 2d). The inflammation was transmural (i.e., endothelial to adventitial) with mixed-inflammatory cell infiltration consisting of numerous macrophages, polymorphonuclear cells, and lymphocytes (Figure 2e, 2f). No fibrinoid necrosis appeared in the area of inflammation. The endothelial lining exhibited a cuboidal ("activated") appearance. In an apparently more progressive case, the wall was replaced by fibroblastic proliferation, collagen deposition, and mixed aggregates of polymorphonuclear cells and lymphocytes (see Figure 2b, asterisk). No alterations in the myocardial tissue were noted in any of the animals. No vasculitis was noted in the other selected organs examined in this study.

In operated mice, IL-6 protein levels in the blood were higher than in unoperated mice, whereas levels of IL-12, IL-2, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) were not different from the nonoperated mice (Table 3).

Balb/c mice have been widely used in genetic, tumor, and immunological studies (Wu et al. 2003) and as an animal model for induction of vasculitis (Luzina and Handwerger 2000; Matsuki et al. 2005; Shepherd et al. 2004). Nevertheless, until now, the spontaneous occurrence of vasculitis in this mouse strain was not regularly reported. In the present series of experiments, we have shown that this mouse strain may have a variable range of spontaneous occurrence of vasculitis restricted to the base of aorta, ranging between 10% and 55%. Because of the high variability of incidence rates between experiments and the different trimming methods used, we did not combine the incidence rates from the two reported experiments.

The aortic root is relatively small in size, and it is often missed by routinely performed heart sectioning. The "multiple-section sampling" method considerably increases the number of aortic root sections available for pathological examination, thus allowing a more reliable assessment of the diagnostic rate. This method, when used in the kidneys, was indeed shown to increase diagnostic rates in carcinogenicity studies (Eustis et al. 1994). In addition, it allowed us a more accurate description of the extent of the lesions, which can range from focal to an annular aspect. We suggest that studies that assess aortitis use this method, especially considering the potentially high sensitivity of the aortic root to mechanical stress (Matsuki et al. 2005).

The induction of acute ischemia may trigger a major inflammatory reaction (Tang et al. 2005), accompanied by the release of several pro-inflammatory cytokines. Significantly higher levels of IL-6 in the operated group have been shown to correlate with increased concentrations in the gastrocnemius muscle after a similar procedure (Tang et al. 2005). In addition, IL-6 is known for its pro-angiogenic role, and the elevation of IL-6 secretion measured in the current study could indicate angiogenic processes following the induced ischemic stress.

Injection of PLX-PAD cells likely did not promote the aortitis, as no treatment-related increase was reported in preclinical safety studies using identical and different mouse strains

(Ramot et al. in press). No treatment-related increases in aortitis or arterial lesions in any other location were noted.

We have confirmed the occurrence of spontaneous aortitis in the Balb/c mouse strain. The presence of background vascular lesions in this mouse strain is of special interest following its use in models for inducing a vascular lesion. Femoral-artery ligation did cause an increase in blood levels of the inflammatory cytokine IL-6, but this finding was not correlated with an increase in the incidence of vasculitis.

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