Original Research

Genetic background determines inflammatory angiogenesis response to dipyridamole in mice

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Abstract

Inflammation and angiogenesis, key components of fibrovascular tissue growth, exhibit considerable variability among species and strains. We investigated whether the response of inbred and outbred mice strains to dipyridamole (DP) on these processes would present similar variability. The effects of the drug on blood vessel formation, inflammatory cell recruitment, collagen deposition and cytokine production were determined on the fibroproliferative tissue induced by sponge implants in Swiss and Balb/c mice. Angiogenesis as assessed by hemoglobin (Hb) and vascular endothelial growth factor (VEGF) concentrations differed between the strains. Swiss implants had the highest Hb content but the lowest VEGF concentrations. Systemic DP treatment exerted an antiangiogenic effect on Balb/c implants but an proangiogenic effect on Swiss implants. The inflammatory enzyme activities myeloperoxidase (six-fold higher in Balb/c implants) and *N*-acetyl-β-D-glucosaminidase were reduced by the treatment in Balb/c implants only. Nitrite concentrations were also higher in Balb/c implants by 40% after DP treatment. Tumor necrosis factor-alpha levels were similar in the implants of both strains and were not reduced by DP. Transforming growth factor β-1 levels and collagen deposition also varied between the strains. The inbred strain had similar levels of the cytokine but implants of Swiss mice presented more collagen. DP treatment reduced collagen deposition in Balb/c implants only. Our data showing the influence of the genetic background on marked heterogeneity of inflammatory angiogenesis components and differential sensitivity to DP may provide some answers to clinical evidence for resistance to angiogenic therapy.

Keywords: cytokine, sponge implant, mouse strain

Experimental Biology and Medicine 2012; **237**: 1084–1092. DOI: 10.1258/ebm.2012.012066

Introduction

Exogenous as well as endogenous factors have been shown to influence the phenotype of a number of physiological and pathological responses in human and experimental animals. Thus, the same stimulus can induce a completely different response when environmental and/or genetic variation is involved. Genetically, the interaction between allelic variants, sequence differences and levels of gene expression are thought to determine and influence a particular phenotype in developmental, physiological and pathological processes.[1–3] In fact, genetic background has been demonstrated to influence spontaneous collateral vessel formation as well as other components (angiogenesis, inflammation and fibrosis) of tissue repair/regeneration.[4–12]

Although these differences present functional and structural implications, there are scarce data addressing the influence of genetic heterogeneity on drug sensitivity on processes where angiogenesis and inflammation co-exist. In one study, dissociation of sensitivities to tumor promotion and progression using 7,12-dimethylbenz[α]-antracene and 12-O-tetradecanoyphorbol-13-acetate have been demonstrated in outbred and inbred mice.[13] More direct evidence of differential responses to angiogenic therapy has been reported by Rohan *et al.*,[14] who showed distinct sensitivities between mouse strains to angiogenesis inhibitors (thalidomide and TNP-470) using a corneal neovascularization model. Similarly, Fukino *et al.*[6] have shown that therapeutic effectiveness of vascular endothelial growth factor (VEGF) to improve ischemia was associated with genetic heterogeneity. We reasoned that other mouse strains might have differential sensitivity to other compounds acting on the vascular system. One such compound, dypyridamole (DP; 2,6-bis[diethanolamino]-4,8-dipiperidinopyrimido-[5,4-d]-pyrimidine), primarily used

as an antiplatelet agent, has been shown to exert antiproliferative effects on rat vascular smooth muscle cells and human mesangial cells.[15–17] In addition, recent evidence has shown angiogenic activity of DP on a mouse hind-limb model.[18] The purpose of this study was to investigate the effects of DP on inflammation, angiogenesis and fibrogenesis induced by sponge implants in Balb/c and Swiss mouse strains, since the temporal pattern and intensity of inflammatory angiogenesis in these mouse strains were clearly shown to be determined by the genetic background.[19] Finding new indications for existing drugs (drug repositioning) is of commercial and medical value, besides a relevant strategy to extend the use of therapeutic compounds for which pharmacodynamics, pharmacokinetics and side-effects have been determined. The results demonstrate that there is a marked heterogeneity in the inflammatory, angiogenic and fibrogenic responses to DP, implying a genetic association with pharmacological sensitivity.

Materials and methods

Animals

Male Swiss and Balb/c mice, 7–8 weeks old (20–30 g body weight), were used in these experiments. The mice were provided by the Central Animal Facility at the Institute of Biological Sciences, Federal University of Minas Gerais-Brazil. The animals were housed individually and provided with chow pellets and water *ad libitum*. The light/dark cycle was 12:12 h with lights on at 7:00 and lights off at 19:00. Efforts were made to avoid all unnecessary distress to the animals. Housing, anesthesia and postoperative care concurred with the guidelines established by our local Institutional Animal Welfare Committee.

Preparation of sponge discs and implantation

Polyether-polyurethane sponge (Vitafoam Ltd, Manchester, UK) was used as the implanted material. The implants were discs, 5 mm thick × 8 mm diameter, and were soaked overnight in 70% v/v ethanol and sterilized by boiling in distilled water for 15 min before implantation. For that, the animals were anesthetized with 2,2,2-tribromoethanol (1 mg/kg; intraperitoneally; Sigma-Aldrich, St Louis, MO, USA), the dorsal hair shaved and the skin wiped with 70% ethanol. The sponge discs were aseptically implanted into a subcutaneous pouch, which had been made with curved artery forceps through a 1-cm long dorsal mid-line incision. Postoperatively, the animals were monitored for any signs of infection at the operative site, discomfort or distress; any showing such signs were immediately humanely killed. DP treatment (200 mg/kg/day by gavage; Boehringer Ingelheim, Ridgefield, CT, USA) started 24 h post sponge implantation and lasted for six days. Control animals received vehicle (drinking water) in the same schedule. The animals were sacrificed seven days after sponge implantation, the implants removed and processed for determination of inflammatory parameters (myeloperoxidase [MPO] and *N*-acetyl-β-D-glucosaminidase [NAG] activities), for assessment of vascularization (hemoglobin [Hb] content, VEGF concentrations and histological analysis), inflammatory cytokines (tumor necrosis factor-alpha [TNF-α] and CCL2), nitrite concentration (indirect evaluation of nitric oxide) and fibrogenic response (collagen and transforming growth factor β-1 [TGF-β1] levels).

Hemoglobin extraction

The extent of the vascularization of the sponge implants was assessed by the amount of Hb detected in the tissue using the Drabkin method.[20,21] Seven days postimplantation, the animals were killed and the sponge implants carefully removed, dissected free from adherent tissue and weighed. Each implant was homogenized using an Ultra-Turrax, (Schlappmuhler, Usingen, Germany) in 5 mL of Drabkin reagent (Labtest, São Paulo, Brazil) and centrifuged at 12,000 × *g* for 20 min. The supernatants were filtered through a 0.22 μm Millipore filter (Danvers, MA, USA). The Hb concentration in the samples was determined spectrophotometrically by measuring absorbance at 540 nm using an enzyme-linked immunosorbant assay (ELISA) plate reader and compared against a standard curve of Hb. The content of Hb in the implant was expressed as μg Hb per mg wet tissue.

Tissue extraction and determination of MPO and NAG activities

The number of neutrophils in implants was measured by assaying MPO activity as previously described.[20,21] The implants were weighed, homogenized in pH 4.7 buffer (0.1 mol/L NaCl, 0.02 mol/L Na_3PO_4, 0.015 mol/L Na_2EDTA) and centrifuged at 12,000 × *g* for 10 min. The pellets were then re-suspended in 0.05 mol/L sodium phosphate buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide followed by three freeze–thaw cycles using liquid nitrogen. MPO activity in the supernatant samples was assayed by measuring the change in absorbance (optical density; OD) at 450 nm using tetramethylbenzidine (1.6 mmol/L) and H_2O_2 (0.3 mmol/L). The reaction was terminated by the addition of 50 μL of H_2SO_4 (4 mol/L). Results were expressed as change in OD/g wet tissue.

The infiltration of mononuclear cells into the implants was quantified by measuring the concentrations of the lysosomal enzyme NAG present in high concentrations in activated macrophages.[20,21] The implants were homogenized in NaCl solution (0.9% w/v) containing 0.1% v/v Triton X-100 (Promega, Madison, WI, USA) and centrifuged (3000 × *g*; 10 min at 4°C). Samples (100 μL) of the resulting supernatant were incubated for 10 min with 100 μL of *p*-nitrophenyl-*N*-acetyl-beta-D-glucosaminide (Sigma-Aldrich) prepared in citrate/phosphate buffer (0.1 mol/L citric acid, 0.1 mol/L Na_2HPO_4; pH 4.5) to yield a final concentration of 2.24 mmol/L. The reaction was stopped by the addition of 100 μL of 0.2 mol/L glycine buffer (pH 10.6). Hydrolysis of the substrate was determined by measuring the absorption at 400 nm. The results were expressed as nmol/mg wet tissue.

Collagen measurement

Total soluble collagen was measured in whole implant homogenates by the Sirius Red (Merck, Darmstadt, Germany) reagent based-assay.[21–23] The implants (6–8 per group) were homogenized in 1 mL of phosphate-buffered saline (PBS) and 50 μL of sample was mixed with 50 μL of Sirius Red reagent. Samples were mixed by gentle inversion. The collagen–dye complex was precipitated by centrifugation at 5000 $\times$ g for 10 min. The supernatants were drained off, discarded and the pellet washed with 500 μL of ethanol (99% pure and methanol free). One milliliter of a 0.5 mol/L NaOH solution was added to the remaining pellet of collagen-bound dye. Following solubilization, samples were transferred to a 96-well plate and read at 540 nm. The calibration curve was set up on the basis of a gelatin standard (Merck). The results were expressed as micrograms of collagen per milligram wet tissue.

Measurement of VEGF, TNF-α, monocyte chemoattractant protein-1 or TGF-β1 production in the sponge implants

Implants removed seven days postimplantation were homogenized in PBS (1 mL for 100 mg of the tissue) pH 7.4 containing 0.05% Tween and centrifuged at 10,000 $\times$ g for 30 min. The cytokines VEGF, TNF-α, monocyte chemoattractant protein 1 (MCP-1) and TGF-β1, in the supernatant from each implant were measured in 50 μL of the supernatant using immunoassay kits (R&D Systems, Minneapolis, MN, USA) and following the manufacturer's protocol. Briefly, dilutions of cell-free supernatants were added in duplicate to ELISA plates coated with a specific murine monoclonal antibody against the cytokine, followed by the addition of a second horseradish peroxidase-conjugated polyclonal antibody against the cytokine. After washing to remove any unbound antibody–enzyme reagent, a substrate solution (50 μL of a 1:1 solution of hydrogen peroxide and tetramethylbenzidine 10 mg/mL in dimethyl sulfoxide) was added to the wells. The color development was stopped, after a 20-min incubation, with 2N sulfuric acid (50 μL) and the intensity of the color was measured at 540 nm on a spectrophotometer (EMax; Molecular Devices, Sunnyvale, CA, USA). The standards were 0.5 $-\log_{10}$ dilutions of recombinant murine cytokines from 7.5 to 1000 pg/mL (100 μL). The results were expressed as picograms of cytokine per mg wet tissue.

Measurement of nitric oxide production

Nitric oxide (NO) release was evaluated by measuring nitrite concentrations according to the method described by Green *et al.*[24] Sponge implants removed seven days postimplantation were weighed and incubated for 15 min at 37°C with PBS (500 μL). The incubation medium (100 μL) was mixed with 10 μL of Griess reagent (0.1% N-1-naphthylediamine, 1% sulfanilamide in 5% H_3PO_4) and optical density was measured at 540 nm. The amount of nitrite in the incubation media was calculated using sodium nitrite (Sigma-Aldrich) as standard.

Histological analysis and staining

The sponge implants from a separate group of mice were excised carefully, dissected free of adherent tissue and fixed in formalin (10% w/v in isotonic saline). Sections (5 μm) were stained with hematoxylin and eosin and processed for light-microscopic studies. Immunohistochemistry reactions for the detection of endothelial cells/blood vessels were performed using the monoclonal antibody clone CD31 (Fitzgerald, Concord, MA, USA). Tissue sections (5 m) were dewaxed and antigen retrieval was performed in citrate buffer (pH 6); the slides were boiled in this buffer for 25 min at 95°C and then cooled for one hour in the same buffer. Sections were incubated for five minutes in 3% hydrogen peroxide to quench endogenous tissue peroxidase. Non-specific binding was blocked by using normal goat serum for 10 min (1:10 in phosphate-buffered saline) with 1% bovine serum albumin (in PBS). The sections were then immunostained with monoclonal antibody to CD31 (1:40 dilution; DAKO Corporation, Carpinteria, CA, USA) for 60 min at room temperature. After washing in Tris–HCl buffer, sections were incubated for 30 min at room temperature with byotinylated Link Universal Streptavidin-HRP (Dako). The reactions were revealed by applying 3,3′-diaminobenzidine in chromogen solution (Dako). The sections were counterstained with hematoxylin and mounted in Permount (Fisher Scientific, Pittsburgh, PA, USA). Immunostaining was performed manually, and spleen was used as positive control. Negative controls were carried out with omission of the primary antibody, resulting in no detectable staining. The expression of these proteins was evaluated on the basis of extent of cytoplasmic immunolabeling in endothelial cells forming lumen in six high-power fields, regardless of staining intensity ($\times$400).

To perform morphometric analysis, images of cross-sections obtained from 15 fields (8533 μm^2) were captured with a plan apochromatic objective ($\times$40) in light microscopy (final magnification = $\times$400). The images were digitized through a JVC TK-1270/JCB microcamera (Tokyo, Japan) and transferred to an analyzer (Carl Zeiss – KS300 version 2, Kontron Electronics, Oberkochen, Germany). A countable vessel was defined as a structure with a lumen containing or not containing red blood cells.

Assessment of cutaneous blood flow in Swiss and Balb/c mice

This was carried out in the dorsal skin of non-implant-bearing mice to establish whether a distinct genetic background would determine variation in this phenotype. We used the fluorimetric method in which the outflow rate of sodium fluorescein applied intradermally indicates local blood flow.[25] A sterile solution (10 μL) of sodium fluorescein (1%; Sigma) was injected intradermally. Blood samples (5 μL) were withdrawn from the tail vein at 1, 3, 5, 7, 10, 15, 20, 25 and 30 min after the dye injection. The blood samples were mixed in 1 mL of isotonic saline, centrifuged for five minutes and the supernatant was kept for fluorescence determination in a Jenway fluorimeter (Model 6200; Essex, UK) at an excitation/emission of

485/520. The results were expressed as the peak of the fluorescence signal in the systemic circulation (min). Five animals of each strain were used to establish this parameter.

Statistical analysis

All data were expressed as mean ± SEM. Comparisons between the groups (numbers as stated in the figure legends; usually 5–8 animals) were made using one-way analysis of variance followed by Newman–Keuls correction factor for multiple comparisons as a post-test. Differences between means were considered significant when P values were <0.05.

Results

Effect of DP on implant-induced angiogenesis in Swiss and Balb/c mice

The sponge matrix was well tolerated by all animals of the two strains. No signs of infection or rejection were observed in the implant location during the seven-day period of the experiment and these implants were easily released from adjacent tissues (skin or muscle). The Hb content of Swiss implants of vehicle-treated animals was about two-fold of Balb/c implants (Figure 1a). DP treatment (200 mg/kg/day) was able to reduce vascularization in Balb/c implants but increased angiogenesis in Swiss implants. The major proangiogenic cytokine (VEGF) was higher in Balb/c implants compared with Swiss implants. DP treatment was able to increase the cytokine in Swiss implants and decrease in Balb/c implants (Figure 1b). The morphometric analysis of the number of blood vessels intraimplant was consistent with the biochemical data showing increased number of neovessels in Swiss implants compared with Balb/c implants and reflected the opposing effect of DP treatment on neovascularization in the two strains (Figure 1c).

Effect of DP on implant-induced inflammation in Swiss and Balb/c mice

Leukocyte accumulation revealed strain-related differences in the profile of the inflammatory response. The difference in neutrophil accumulation (as MPO activity) in the implants of Balb/c mice was six-fold higher compared with Swiss implants (Figure 2a). DP treatment was effective in decreasing this activity in Balb/c implants but failed to change this parameter in implants of Swiss animals (Figure 2a). NAG activity was also decreased in Balb/c implants by the treatment but not in the Swiss implants (Figure 2b). Concentrations of nitrite were similar in both strains but DP treatment was able to increase nitrite production in Balb/c implants (Figure 2c).

The proinflammatory cytokine, TNF-α, showed a similar profile and response in both strains. The treatment did not reduce the cytokine production intraimplant (Figure 3a). Both strains differed in their production of CCL2/MCP-1 and the treatment was ineffective in altering the concentrations of this chemokine (Figure 3b).

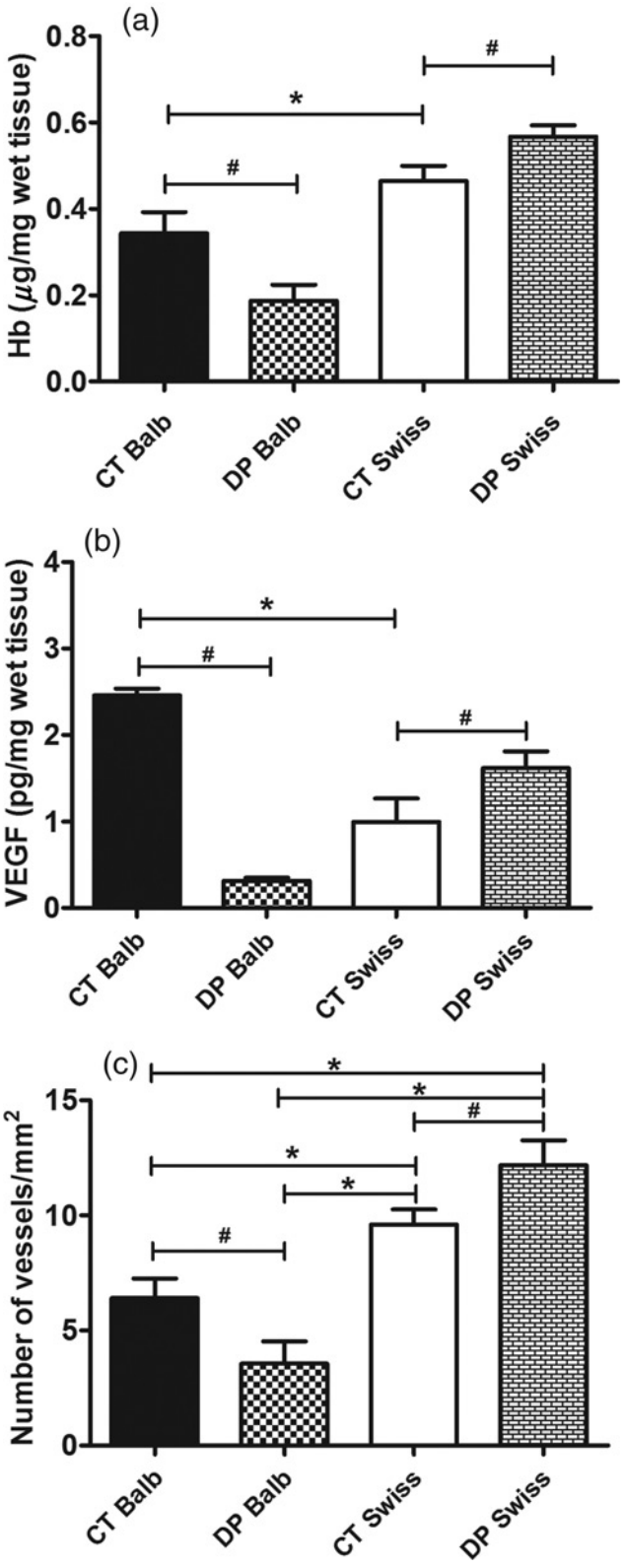

Figure 1 Effect of dipyridamole (DP) on angiogenic parameters in Balb/c and Swiss implants: (a) hemoglobin content; (b) vascular endothelial growth factor production; (c) morphometric analysis of the number of blood vessels intraimplant. Differences in angiogenic parameters and response to DP were observed between both strains. Values shown are the means (± SEM) from groups of 6–8 animals for each group. *Significant difference between Swiss and Balb/c implants (*$P < 0.05$), analysis of variance (ANOVA); #significant difference after DP treatment (#$P < 0.05$), ANOVA. CT, control group

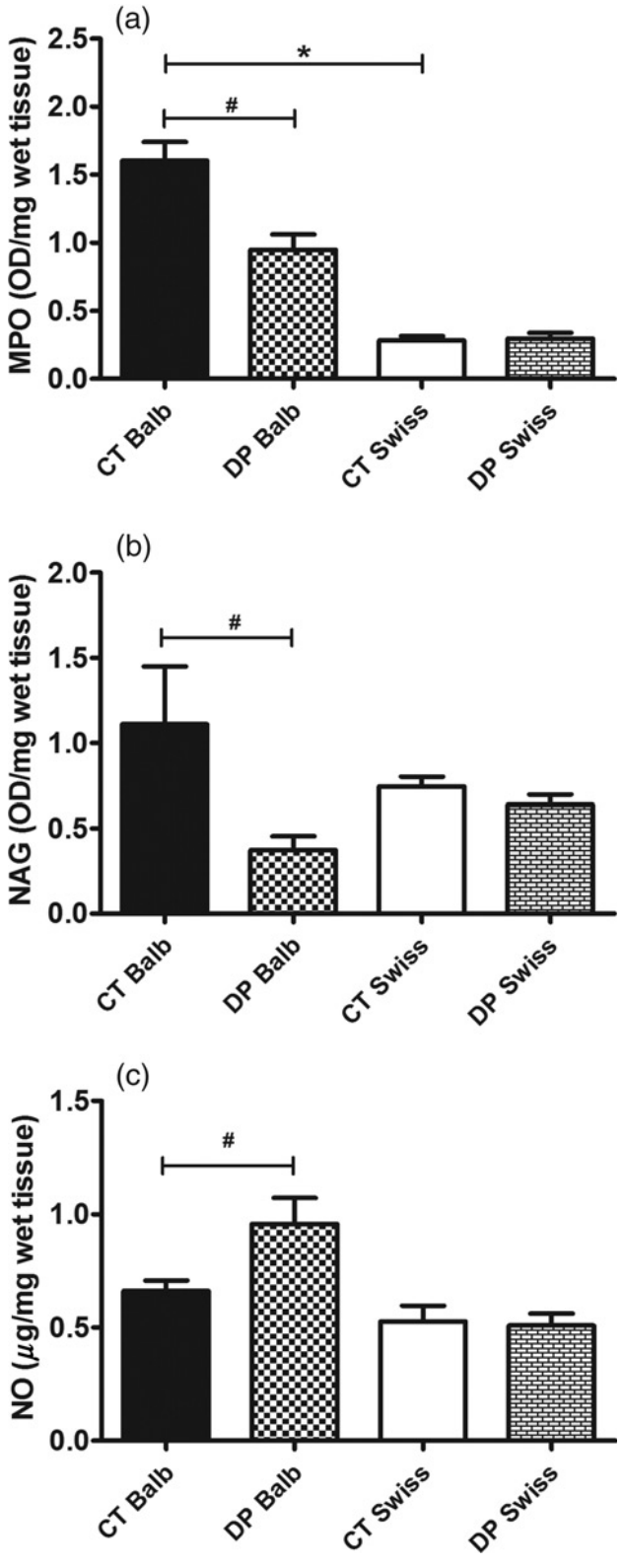

Figure 2 Effect of dipyridamole (DP) on inflammatory parameters in Balb/c and Swiss implants: (a) concentration of neutrophil accumulation (measured as myeloperoxidase [MPO] activity); (b) level of macrophage accumulation; and (c) nitrite production. Values shown are the means (±SEM) from groups of 6–8 animals for each group. *Significant difference between Swiss and Balb/c implants ($^{*}P < 0.05$), analysis of variance (ANOVA); #significant difference after DP treatment ($^{\#}P < 0.05$), ANOVA. NAG, *N*-acetyl-β-D-glucosaminidase; NO, nitric oxide; CT, control group

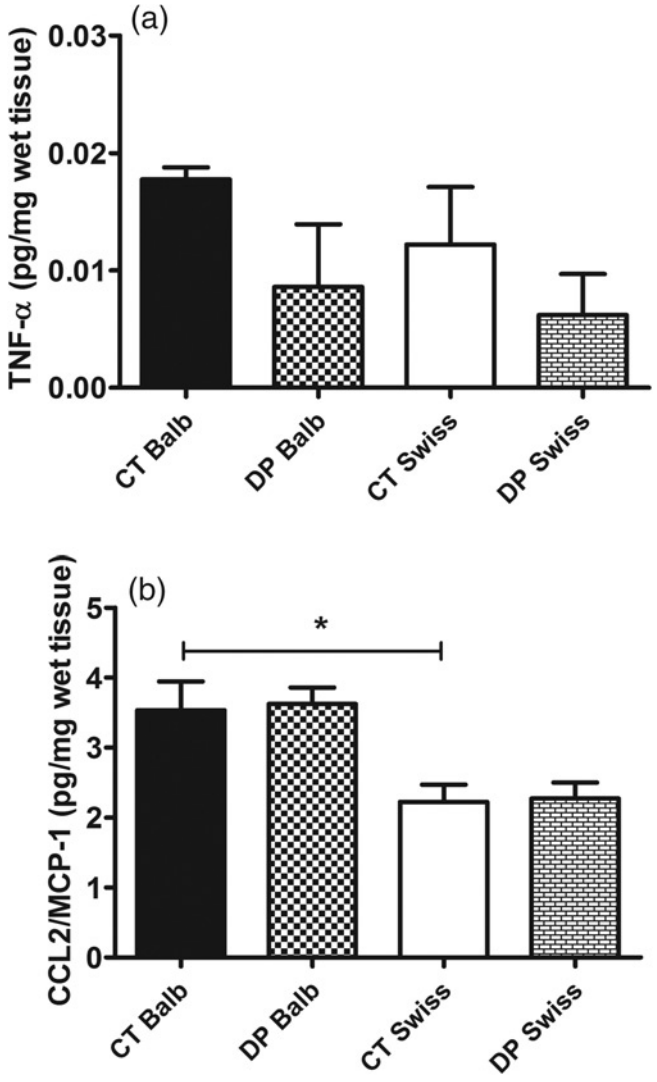

Figure 3 Effects of dipyridamole (DP) on proinflammatory cytokine production in Balb/c and Swiss implants: (a) tumor necrosis factor-alpha (TNF-α) and (b) CCL2(MCP-1/JE). The levels of TNF-α did not vary between the strains, but those of CCL2(MCP-1) were higher in Balb/c implants. Values shown are the means (±SEM) from groups of 6–8 animals at each time point. *Significant difference between Swiss and Balb/c implants ($^{*}P < 0.05$), analysis of variance (ANOVA); #significant difference after DP treatment, ANOVA. CT, control group

Effects of DP on TGF-β1 concentrations and collagen deposition in implants of Swiss and Balb/c mice

The concentrations of the profibrogenic cytokine, TGF-β1, did not differ between the strains but Swiss implant levels of this cytokine increased after DP treatment (Figure 4a). The collagen content was naturally higher in Swiss implants compared with Balb/c implants. DP treatment was able to reduce the collagen level in Balb/c implants but not in Swiss implants (Figure 4b).

Histological analysis of implants from Balb/c and Swiss mice

Histological sections (hematoxylin and eosin staining) of the fibrovascular tissue induced by subcutaneous sponge implants of the two different strains of mice showed

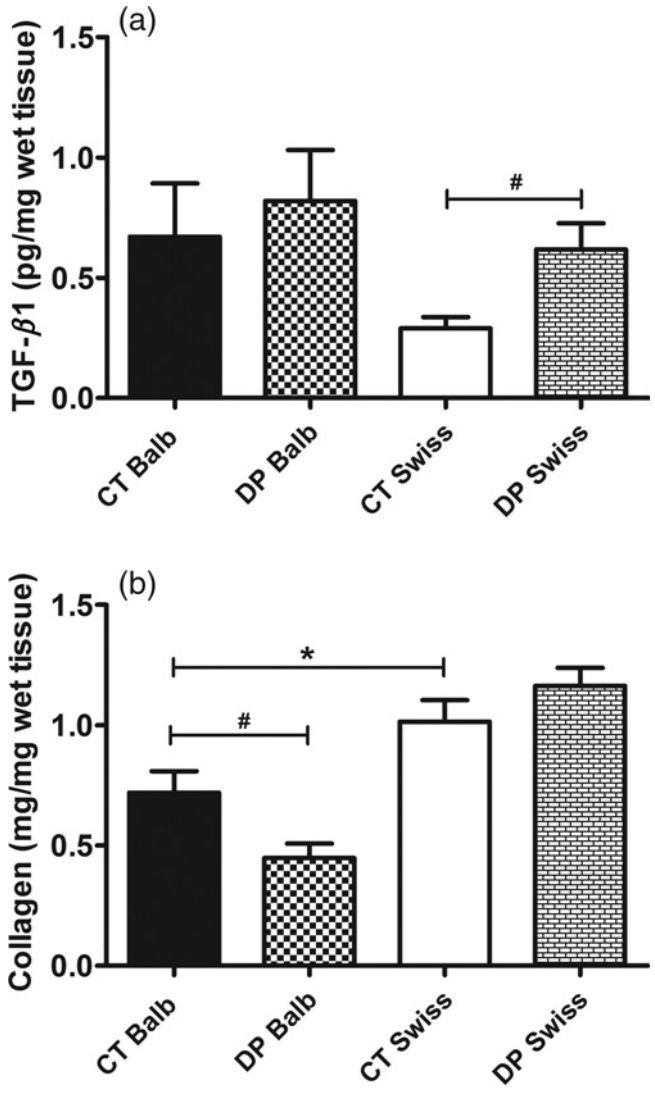

Figure 4 Effects of dipyridamole (DP) on transforming growth factor β1 (TGF-β1) (a) levels and collagen deposition (b) in Balb/c and Swiss implants. Collagen deposition varied between the strains and DP treatment showed strain-related sensitivity. Values shown are the means ($\pm$SEM) from groups of 6–8 animals for each group. *Significant difference between Swiss and Balb/c implants ($^{*}P < 0.05$), analysis of variance (ANOVA); #significant difference after DP treatment ($^{\#}P < 0.05$), ANOVA

a stroma occupying the pores of the synthetic matrix. Blood vessels, inflammatory cells and spindle-shaped fibroblasts were seen in all sections. There were more blood vessels and collagen deposition in Swiss implants compared with the Balb/c implants. In addition, DP treatment was able to reduce inflammation, angiogenesis and collagen deposition in Balb/c implants but not in Swiss implants (Figure 5a–d).

Vasodilator effect of DP on cutaneous blood flow in Swiss and Balb/c mice

Having established that DP exerted distinct modulatory effects on the inflammatory, angiogenic and fibrogenic components of the fibrovascular tissue induced by the implants in Balb/c and Swiss mice, we investigated the classical vasodilator effect of the compound on the skin blood flow of both strains. A single dose of DP (200 mg/kg) given 24 h before blood flow determination was able to decrease the time taken for the fluorescence to peak in the systemic circulation (increase diffusion of fluorescent marker) significantly earlier in both strains compared with the peak of non-treated animals. The results also show that Swiss mice have higher basal fluorescein diffusion compared with Balb/c animals (Figure 6). Thus, although Balb/c and Swiss mice responses to DP were similar (vasodilation), the skin vasculature of Swiss mice was inherently more permeable to the fluorescent marker than that of Balb/c animals.

Discussion

These experiments demonstrate that genetic heterogeneity influences both the development of the components of the fibrovascular tissue as well as the animal response to DP. This compound, a long-lasting, clinically used drug, besides its beneficial effects on a range of vascular conditions, has been shown to exert other biological activities such as anti-inflammatory, antioxidant, antiproliferative, antifibrogenic and antiphosphodiesterase.[16,17,26–28] In addition, it has been demonstrated that DP augments ischemic tissue perfusion, angiogenesis and arteriogenesis due to its ability to increase NO bioavailability.[18] The experimental evidence that DP exerts other therapeutic actions besides those primary described is of commercial and medical value in drug repositioning (new indication for an existing drug). Here, we identified novel properties of the compound and the influence of genetic background in responding to DP.

It is well documented that sponge implantation induces a spatial and temporal localized fibrovascular tissue growth whose components (inflammation, angiogenesis and fibrogenesis) are necessary responses to injury.[20,21] However, in several pathological conditions (chronic inflammation, adhesion and tumor growth), these components are thought to be responsible for maintenance and aggravation of the diseases.[29] By using the implantation technique to evaluate the effect of DP on inflammatory cell recruitment, neovascularization and fibrogenic response of Swiss and Balb/c mice strains, we showed that these parameters and responsiveness to the compound are traits associated with the genetic background. First, because implants of Swiss mice were inherently more vascularized than Balb/c implants. Second, DP was able to decrease angiogenesis in Balb/c implants but increased Hb content and number of vessels of Swiss implants. The angiogenic effect of DP in Swiss implants is in agreement with the proangiogenic effect of the compound in ischemic limbs,[18] but it is in contrast with the antiangiogenic effect in Balb/c mice. Knowing mouse strain differences in responding to potential therapeutic compounds might be relevant for defining the strain choice for drug trials. Another point of discrepancy between the strains was the fact that VEGF concentrations intraimplant (a marker of angiogenesis) were lower in Swiss than in Balb/c implants. Furthermore, DP treatment stimulated VEGF production in Swiss implants but

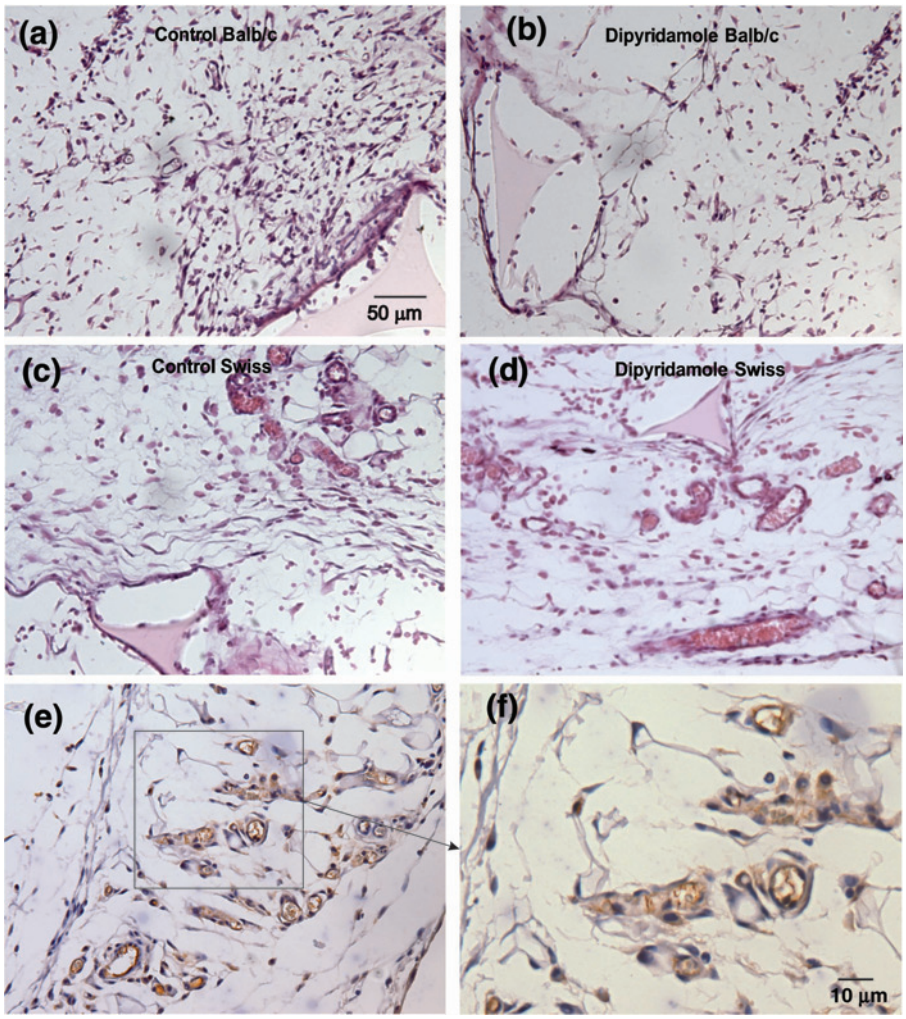

Figure 5 Representative histological sections (5 μm, stained with hematoxylin and eosin and with CD31 monoclonal antibody (e) and (f)) of fibrovascular tissue induced in subcutaneous sponge implants in Balb/c (a) and Swiss (b). The fibrovascular stroma that occupies the pores of the sponge matrix (triangular shapes) is composed of blood vessels, inflammatory cells and spindle-shaped fibroblasts. The tissue in the Swiss implants is more vascularized. Dipyridamole treatment changed the pattern of cellular infiltration in Balb/c implants but not in Swiss implants. Bar: 50 μm; magnification $\times$400. Inset $\times$100, Bar: 10 μm. (A color version of this figure is available in the online journal)

decreased in Balb/c implants. The results indicate marked heterogeneity in cytokine production between both strains and differential sensitivity to DP. The fact that the VEGF concentrations were higher in the least vascularized implants (inbred strain) may indicate an attempt to compensate for this intrinsic blood vessel deficiency. It may be pertinent to link our findings with those by Fukino *et al.*,[6] who demonstrated impaired collateral vessel formation in Balb/c mice compared with another inbred strain (C57BL/6J). Our morphometric analysis of the implants of treated and non-treated animals corroborated the biochemical parameters used as indirect evidence of angiogenesis.

The inflammatory parameters were also a trait associated with the genetic background. MPO activity (a marker of neutrophil recruitment) was approximately six-fold higher in Balb/c implants compared with Swiss implants and NAG activity (a marker of macrophage recruitment) was reduced by the treatment in Balb/c implants. After treatment, Balb/c implants showed more nitrite than Swiss mice. By decreasing the concentrations of the inflammatory enzymes with DP treatment in Balb/c implants, we confirmed the anti-inflammatory effects of the compound described in other animals[26] and disclosed further differences between the strains since implants of Swiss mice were not affected by the treatment. Another interesting finding was that Balb/c mice produced more nitrite that Swiss implants. The effect of DP increasing nitrite production intraimplant in the inbred strain is in agreement with the findings of Patillo *et al.*,[18] who showed an increase in NO/nitrite concentrations after DP treatment in ischemic muscle in C57BL/6J mice (inbred strain). The level concentrations of the proinflammatory cytokines also showed, to some extent, a strain-related difference, that being the levels of TNF-α did not alter in Balb/c and Swiss implants after the treatment and the levels of CCL2/MCP-1, although higher in inbred mice, were not affected by DP. Overall, our results showed that the inbred strain presented a more inflammatory response to the implants and sensitivity to DP whereas the outbred strain was either less sensitive or defective in its inflammatory responses to DP.

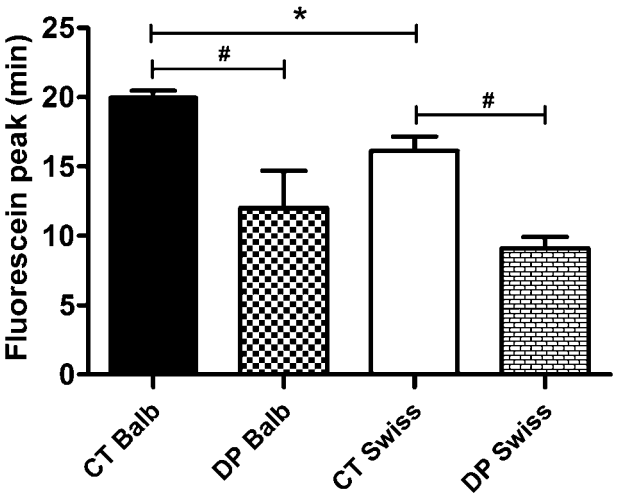

Figure 6 Effect of dipyridamole (DP) on fluorescein diffusion after intradermal injection of the dye in Balb/c and Swiss mouse strains. Values shown are the means (± SEM) from groups of 4–6 animals. *Significant difference between Swiss and Balb/c implants ($^{*}P < 0.05$), analysis of variance (ANOVA); #significant difference after DP treatment ($^{\#}P < 0.05$), ANOVA

The fibrogenic response induced by the implants presented differences between the strains and sensitivity to DP. More collagen deposition and less TGF-β1 production were traits of Swiss implants compared with Balb/c. DP treatment exerted opposing actions on collagen deposition in the two strains; decreased collagen in the inbred strain but increased in outbred mice. The findings reported by Kolb *et al.*[11] showed that Balb/c mice were resistant to lung fibrosis induced by various compounds compared with C57BL/6 mice, suggesting genetic heterogeneity.

After determining variations in key components of the newly formed fibroproliferative tissue and the distinct responses to DP in the two strains of mice as detected by biochemical parameters and corroborated by histological evidence, we investigated if differences would be detected in pre-existing normal tissue. The assessment of cutaneous blood flow using the fluorescein diffusion method[25] revealed that the dye diffusion in Balb/c mice was slower than in the other strain of mice, an inherent functional difference in the skin vasculature of these animals. However, the vasodilator effect of DP was observed in both strains.

Our data showing the influence of the genetic background on marked heterogeneity of inflammatory angiogenesis components and differential sensitivity to DP may provide some answers to clinical evidence for individual resistance to angiogenic therapy. Furthermore, these findings suggest that genetic factors determine individual fibroproliferative potential and response to drugs acting on the process. Altogether, our findings may be relevant to direct the choice of experimental animals or human populations in angiogenic/inflammatory drugs in clinical trials.

Author contributions: All authors participated in the design, interpretation of the results, analysis of the data and review of the manuscript. FPS, SMM, PRC and PPC conducted the experiments and MANDF and SPA wrote the manuscript.

ACKNOWLEDGEMENTS

This work was supported by CNPq and FAPEMIG-Brazil.

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(Received February 16, 2012, Accepted May 21, 2012)