Biomedicine & Pharmacotherapy 86 (2017) 715–724

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Original article

Murine strain differences in inflammatory angiogenesis of internal wound in diabetes

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ARTICLE INFO

Article history:
Received 1 November 2016
Received in revised form 30 November 2016
Accepted 30 November 2016

Keywords:
Angiogenesis
Diabetes
Genetic background
Internal wound

ABSTRACT

Genetic susceptibility is associated with inflammation, neovascularization, and diabetes phenotypes. However, to what extent this susceptibility influences inflammatory angiogenesis in internal injuries in diabetes has not been fully investigated. Using the subcutaneous implantation of a synthetic matrix as an internal wound model in Swiss, C57BL/6 and Balb/c mice, we have studied inflammation, angiogenesis, and cytokine production in the fibrovascular tissue induced by implants in diabetic animals. The hyperglycemic levels (mg/dl) after the diabetogenic treatment were 455.0 ± 15 in Swiss, 393.0 ± 22 in C57BL/6, and 190.0 ± 10 in Balb/c mice. Angiogenesis in Swiss implants from non-diabetic animals were higher than those in the implants from the other strains. However, the angiogenic inducers VEGF and nitric oxide (NO) were higher in implants from non-diabetic Swiss and Balb/c mice. Strain-related differences were also observed in the angiogenic parameters in implants from diabetic mice. Hb content and number of vessels decreased more than 40% in Swiss implants. In contrast, Hb content did not alter in implants from Balb/c diabetic mice and the number of vessels decreased. VEGF levels increased in implants from Swiss and C57BL/6 diabetic mice, but decreased in Balb/c implants. The levels of pro-inflammatory markers intra-implant also varied among the strains in both conditions. In the hyperglycemic environment, almost all inflammatory markers increased in implants from diabetic Swiss mice. These findings demonstrate the major contribution of genetic background in the pattern of inflammatory angiogenesis components of internal injury, in both normoglycemic and hyperglycemic animals.

1. Introduction

Diabetes, a group of metabolic disorders, has common underlying mechanisms associated with hyperglycemia. The duration and intensity of hyperglycemia have been strongly correlated with the rate and progression of diabetes complications (vasculopathy, neuropathy) in both type 1 and type 2 diabetes. The extent of the disease severity and complications varies in each individual, suggesting that genetic background is a relevant factor in the pathological process. In fact, a number of animal studies have demonstrated that mouse strains can differ in their metabolic phenotype. For example, Kooptiwut et al. [1] showed differences in insulin secretory function in two mice models with distinct susceptibility to beta-cell failure. Kulkarni et al. [2] showed the impact of genetic background on development of hyperinsulinemia and diabetes in distinct mice strain. Bock et al. [3] reported the influence of genetic background on the size and structure of endocrine pancreas (variation in islet mass, beta-cell mass, and islet number). Regarding the susceptibility to the diabetogenic treatment (streptozotocin) the mouse strains show inherent differences to the effects of this compound in the development of hyperglycemia [4–6]. Thus, while much is known about the impact of genetic background on constitutive parameters of the metabolic system in diabetes (glucose metabolism, insulin secretion, and disease susceptibility), information on the influence of genetic background on key components (inflammation and

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http://dx.doi.org/10.1016/j.biopha.2016.11.146
0753-3322/

angiogenesis) of healing processes in the hyperglycemic environment is scarce. Interestingly, these processes have been shown to be influenced by a number of factors, including the genetic trait, in the most frequently used experimental mouse strains (Swiss, C57BL/6, Balb/c, DBA) and models of inflammation and neovascularization. For instance, strain-related differences in angiogenesis signaling in response to hypoxia and to angiogenic factors have been reported [7–9]. We have also demonstrated that the genetic background of Swiss, C57BL/6 and Balb/c mice not only influenced the kinetics of sponge-induced inflammatory angiogenesis, but also the response of distinct mouse strains to pharmacological compounds [10,11].

It has been clearly shown that the diabetes-associated healing impairment, which has been extensively investigated in cutaneous wounds, is characterized by a decreased inflammatory response, amount of fibrosis or fibrogenesis, collagen synthesis, tensile strength, angiogenesis, and altered production of cytokines [12–15]. This pattern is in marked contrast with the healing process in non-diabetic individuals, which is characterized by efficient inflammatory cell recruitment, adequate cytokine production, angiogenesis, matrix formation, and reepitheliazation [16,17]. However, less is known about the healing process of internal injuries in diabetes, as pointed out by Le et al. [18]. In a limited number of studies, using subcutaneous implants in rabbits, baboons, and rats, the formation of granulation tissue and connective tissue ingrowth within and around the devices in diabetic animals were shown to be impaired [19,20]. Although it is thus clear that the angiogenic and inflammatory components of repair processes in diabetes are impaired, no direct comparison of these parameters has been reported in Swiss, C57BL/6 and Balb/c mice. Therefore, our aim in this study to was to determine the effect of mouse strain on the pattern of the inflammatory and angiogenic components of internal fibroproliferative tissue induced by synthetic matrix in diabetic mice. An analysis of the components of the newly formed proliferating fibrovascular tissue might disclose whether genetic background would influence the defective inflammation and angiogenesis in repair processes in diabetic animals. This information would be particularly relevant when considering the animal model of diabetes and therapeutic approaches in healing deficiencies in diabetic individuals.

2. Material and methods

2.1. Animals

All animal care and experimental procedures complied with the guidelines established by our local institutional animal welfare committee. Efforts were made to avoid unnecessary distress to the animals. Female mice Swiss, C57BL/6, and Balb/c were divided into six groups of 10 animals each for biochemical analyses and 5 of each group for histological analyses. The animals were 8–10 weeks old and 25–30 g body weight. The mice were provided by the Central Animal Facility at the Institute of Biological Sciences, Federal University of Minas Gerais number 275/2014. The animals were housed individually and provided with chow pellets and water ad libitum. The light/dark cycle was 12 h/12 h with lights on at 7:00 a.m. and lights off at 7:00 p.m.

2.2. Induction of diabetes mellitus

Streptozotocin (STZ) was obtained from Sigma-Aldrich, St. Louis, MO, USA. STZ was dissolved in a 10 mM sodium citrate buffer, pH 4.5 and always prepared for immediate use within 5–10 min. STZ doses were determined according to the body weight of animals and administered intravenously in injections of 50 mg/kg for 5 consecutive days. This experimental protocol has been shown to induce a model of insulin insufficiency and diabetes type 1 [6,21,22]. The glucose concentration in the blood was measured in fasted state (8 h) in all animals through blood samples taken from the tail vein before the first dose of streptozotocin and on the 20th day after the injections. The measurement was performed using a glucometer Call® On Plus Blood Glucose Meter (ACON Laboratories, Inc.). Animals whose blood glucose levels exceeded 180 mg/dl after treatment were considered diabetic.

2.3. Preparation of sponge discs and implantation

Polyether–polyurethane sponge discs, 5 mm thick × 8 mm diameter (Vitafoam Ltd., Manchester, U.K.) were used as the matrix for fibrovascular tissue growth as previously described [23,24]. The sponge discs were soaked overnight in 70% ethanol and sterilized by boiling in distilled water for 30 min before the implantation surgery. Fifteen days after the last dose of STZ injection following confirmation of diabetes, the animals were anesthetized with a 40 μL mixture of ketamine and xylazine (57 mg/ml and 8.6 mg/ml, respectively). The dorsal hair was shaved and the skin wiped with 70% ethanol. The sponge discs were aseptically implanted inside a subcutaneous pouch, which had been made with curved artery forceps through a 1 cm long dorsal mid-line incision. The incisions were closed with silk braided non-absorbable suture. At 10 days post implantation (25 days after induction of diabetes), the animals were anesthetized with ketamine and xylazine and later killed by cervical dislocation. At this point, the fibrovascular tissue induced by the sponge matrix is composed of well-developed blood vessels containing red blood cells, inflammatory cells, fibroblasts within a mature extracellular matrix. All these features are present in the granulation tissue during healing processes.

The sponge discs were carefully dissected from adherent tissue, removed and weighed. They were then processed as described below for the various assays.

2.4. Hemoglobin extraction

The extent of vascularization of the sponge implants was assessed by the amount of hemoglobin (Hb) detected in the tissue using the Drabkin method. At 10 days post implantation, the animals were killed and the sponge implants carefully removed, dissected, cleared of any adherent tissue, and weighed. Each implant was homogenized (T10 basic Ultra-Turrax disperser; S10 N – ST dispersing element, IKA) in 2 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. The hemoglobin concentration in the samples was determined spectrophotometrically by measuring absorbance at 540 nm using an ELISA plate reader and comparing it against a standard hemoglobin curve. Hemoglobin content in the implant was expressed as μg Hb per mg wet tissue [10,21].

2.5. Tissue extraction and determination of myeloperoxidase (MPO) and N-acetyl-β-D glucosaminidase (NAG) [25] activities

The number of neutrophils in implants was measured by assaying myeloperoxidase (MPO) activity as previously described [22,26]. The implants were weighed, homogenized in saline sodium phosphate EDTA-HCl buffer (0.1 M NaCl, 0.02 M Na_3PO4, 0.015 M Na_2EDTA; using HCl to adjust pH), pH 4.7, and centrifuged at 12,000*g* for 20 min, 4 °C. The pellets were then re-suspended in 2 ml of 0.05 M sodium phosphate buffer (pH 5.4) containing 0.5% hexa1,6-bis-decyltrimethylammonium bromide (HTAB, Sigma), homogenized for 30 s, followed by three freeze–thaw cycles using liquid nitrogen. MPO activity in the supernatant samples was