Environmental Toxicology and Pharmacology 54 (2017) 93–98

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

Oral warfarin intake affects skin inflammatory cytokine responses in rats

Aleksandra Popov Aleksandrov[a,1], Ivana Mirkov[a,1], Lidija Zolotarevski[b], Marina Ninkov[a], Dina Mileusnic[a], Dragan Kataranovski[a,c], Milena Kataranovski[a,d,*]

[a] Immunotoxicology Group, Department of Ecology, Institute for Biological Research "Sinisa Stankovic", University of Belgrade, Belgrade, Serbia
[b] Medical College of Applied Sciences, Belgrade, Serbia
[c] Institute of Zoology, Faculty of Biology, University of Belgrade, Belgrade, Serbia
[d] Institute of Physiology and Biochemistry, Faculty of Biology, University of Belgrade, Belgrade, Serbia

ARTICLE INFO

Keywords:
Rats
Oral warfarin intake
Skin
Epidermal cells
Dinitrochlorobenzene
Inflammatory cytokines
Interleukin-10

ABSTRACT

Warfarin is an anticoagulant used in prevention/prophylaxis of thromboembolism. Besides the effects on coagulation, non-hemorrhagic reactions have also been documented. Although cutaneous reactions were reported in some patients, the impact on skin immunity was not explored. In the present paper, the effect of 30-day oral warfarin intake on skin cytokine responses in rats was analyzed. Increased release of inflammatory cytokines (TNF, IL-1β and IL-10) was noted by skin explants from rats which received warfarin, but without effect on IL-6. No impact on epidermal cell cytokine secretion was seen, except a tendency of an increase of IL-6 response to stimulation with microbial product lipopolysaccharide (LPS). Topical application of contact allergen dinitrochlorobenzene (DNCB) resulted in slight (numerical solely) increase of TNF release by skin explants of warfarin-treated animals, while epidermal cells responded by increased secretion of all four cytokines examined. The data presented provide new information on the potential of oral warfarin to modulate skin innate immune activity.

1. Introduction

Although in use for more than 60 years, warfarin is still the most widely used anticoagulant drug in the world (Pirmohamed, 2006). Its use (according to prescriptions data) increased by 45% from 1998 to 2004 (Aguilar and Hart, 2005). Warfarin affects interconversion of Vitamin K (VK) and its 2,3 epoxide by inhibiting vitamin K epoxide reductase (VKOR). As a consequence of VKOR inhibition, depletion of hydroquinone form of VK (K1H2) (a cofactor of γ-glutamyl carboxylase which enables carboxylation of glutamyl (Gla) residues on precursors of several proteins involved in hemostasis) occurs and undercarboxylated, biologically non-active blood coagulation factors are generated including factor II (prothrombin, PT), factor VII (FVII), factor IX (FIX) and factor X (FX) (Furie, 2000). Because of narrow therapeutic index, it is difficult to maintain defined range of anticoagulation in patients and there is a risk of bleeding (due to warfarin overdose) or thrombotic events (at too low warfarin dose) (Boulanger et al., 2006).

According to data, in Great Britain since 2004, warfarin has reached the third position on the list of drugs resulting in admission to hospital because of its adverse effects (Pirmohamed et al., 2004). The major undesired effect of warfarin is bleeding (Linkins et al., 2003). Major bleeding is the most important clinical manifestation of oral warfarin therapy which affects a variety of tissues and organs (Linkins et al., 2003) including central nervous system (Flaherty et al., 2007). Non-hemorrhagic adverse effects of warfarin were also reported and skin was recognized as one of the affected tissues (Gallerani et al., 1995).

Warfarin-induced skin necrosis (WISN) is a rare (0.01%–0.1%) clinical complication in patients on oral warfarin therapy (Chan et al., 2000). Cutaneous hemorrhage with vascular damage and thrombosis with skin necrosis is the typical histological feature in these patients (Ad El et al., 2000). Signs of immune activity (proinflammatory cytokine TNF in necrotic tissue and endothelial adhesion molecule at the periphery of the lesion) were described in some patients (Hermes et al., 1997). Deficiency/low levels of proteins C and S, as well as antithrombin III deficiency are considered as major risk factors for WISN (Nazarian et al., 2009). Besides WISN, other adverse health effects of oral warfarin therapy were reported including hypersensitivity reactions such as ecchymoses and purpura, skin eruptions (vesicular and maculopapular), purple toes and exanthematous reactions (Bircher et al., 2006; Kakagia et al., 2014; Nazarian et al., 2009; Panuncialman and Falanga, 2010). Hypersensitivity to different coumarins was also described (Kruis-de Vries et al., 1989) and positive response to re-

* Corresponding author at: Immunotoxicology Group, Department of Ecology, Institute for Biological Research "Sinisa Stankovic", University of Belgrade, 142 Bulevar despota Stefana, 11000 Belgrade, Serbia.
E-mail address: milena@ibiss.bg.ac.rs (M. Kataranovski).
[1] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.etap.2017.06.027
Received 16 December 2016; Received in revised form 15 May 2017; Accepted 26 June 2017
Available online 28 June 2017

challenge was noted (Spyropoulos et al., 2003). Warfarin-induced leukocytoclastic vasculitis (LCV) was rarely reported and only several cases were described (Howitt et al., 1982; Hsu et al., 2012; Jiménez-Gonzalo et al., 1999; Susano et al., 1993; Tamir et al., 1994; Tanay et al., 1982; Yaghoubian et al., 2005). It could be associated with skin necrosis or as a separate entity (Kurt et al., 2007). Most common histological finding of involved skin are structural changes of dermal vasculature (sometimes with fibrinoid necrosis) and perivascular infiltration of polymorphonuclear/neutrophil and mononuclear cells. Oral warfarin also causes systemic hypersensitive reactions characterized by eosinophilia (Goudarzipour et al., 2015; Hall and Link, 1981; Jo et al., 2011; Kuwahara et al., 1995; Teragaki et al., 2012) and presence of eosinophils was noted in the skin and kidneys in some patients (Kapoor and Bekaii-Saab, 2008). All these studies showed that cutaneous effects of oral warfarin therapy had involved immune system cells, but there were no data concerning effects on components of regional skin immune system in such settings.

Using a model of repeated epicutaneous administration of warfarin in rats (Kataranovski et al., 2007, 2003; Popov Aleksandrov et al., 2015) it has been shown that skin is a target organ for immunomodulatory effects of this agent. Influence of 30-days oral warfarin intake on basic aspects of peripheral blood leukocyte activity in rats (Belij et al., 2012; Popov et al., 2013) at doses which resulted in visceral organ hemorrhage (Mirkov et al., 2016) depicted immunomodulatory effects of this agent when received as oral treatment.

In view of these and above cited data, the aim of the present paper was to examine whether oral warfarin intake exerted influence on immune/inflammatory activity in the skin. Therefore, the effect of 30-day oral warfarin intake on the skin and epidermal cell inflammatory cytokine (TNF, IL-1β and IL-6) response was examined in rats. Because IL-10 is involved in the inflammatory responses of keratinocytes, this cytokine was measured too. The impact of warfarin on responsiveness to external stimulation by skin sensitizer dinitrochlorobenzene (DNCB) was examined as well. Data obtained showed stimulatory effects of warfarin on skin inflammatory cytokine responses, which is a novel finding.

2. Materials and methods

2.1. Chemicals

Warfarin sodium was purchased from Serva Fein Biochemica (Heidelberg, Germany). Dispase II was obtained from Boehringer (Manheim, Germany) and trypsin solution from Difco (Lawrence, KS, USA). Hexadecyltrimethylammonium bromide (HTAB), *o*-dianisidine dihydrochloride, myeloperoxidase (MPO), three-(4,5-dimethyl-thiazol-2-yl)-2,5 diphenyl-tetrazolium bromide (MTT), lypopolysaccharide (LPS) and phenylmethanesulfonyl fluoride (PMSF) were all purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). 1-Chloro-2,4-dinitrochlorobenzene (DNCB) was obtained from BDH Chemicals Ltd., London, UK and dissolved in 4:1 acetone:olive oil (vehicle). Hydrogen peroxide (H_2O_2) was purchased from Zorka Farma (Sabac, Serbia). Dispase II was dissolved in culture medium. All solutions for cell culture experiments were either prepared under sterile conditions or were sterile filtered (Flowpore, pore size 0.22 μm) before use. Culture medium RPMI-1640 (Biowest, Nuaillé, France) supplemented with 2 mM glutamine, 20 μg/ml gentamycine (Galenika a.d., Serbia), 5% (v/v) heat inactivated fetal calf serum (Biowest, Nuaillé, France) was used in cell culture experiments.

2.2. Animals

All procedures used herein complied with the European Communities Council Directive (86/609/EEC) and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research “Sinisa Stanković” (IBISS), Belgrade, Serbia. Male Dark Agouti (DA) rats eight to ten weeks old, conventionally housed at the IBISS animal facility were used. Eight animals were assigned to each treatment group. All analyses were done in samples of eight animals.

2.3. Warfarin treatment

Warfarin sodium was prepared in drinking water at concentration of 3.5 mg/l and was given to rats for 30 days. Warfarin was replaced with freshly prepared solution twice a week. Control animals received water solely. The effectiveness of warfarin intake was checked by measurements of prothrombin time (PT) and partial thromboplastin time (PTT) (described below), which are the biological markers of the anticoagulant effect of warfarin.

2.4. Prothrombin time (PT) and partial thromboplastin time (PTT)

Prothrombin time (PT) and partial thromboplastin time (PTT) were determined in blood samples obtained from abdominal artery and diluted with 3.8% sodium citrate (final blood to citrate ratio of 5:1). PT was determined by one-stage method using citrate plasma and Thromborel S reagents (Behring Diagnostics GmbH, Marburg, Germany) with Siemens equipment. The kaolin-activated PTT was determined by one stage method using Pathrombin (Behring).

2.5. Histology

Skin samples were taken 24 h following completion of warfarin intake. They were fixed in 4% buffered formaldehyde (pH 6.9) and embedded in paraffin wax for sectioning at 5 μm. Hematoxylin and eosin (H & E)-stained histology slides were subsequently analyzed by a certified histopathologist in a blinded manner using a Coolscope digital light microscope (Nikon Co, Tokyo, Japan).

2.6. Skin explants culture

Skin was excised 24 h following completion of warfarin intake, cleared of subcutaneous tissue, cut into pieces (~10 × 10 mm) and placed in duplicate of 0.4 ml complete culture medium. After 48 h culture supernatant (conditioned medium) was collected by centrifugation.

2.7. Epidermal cell preparation and culture

Skin was cut into small pieces and incubated in dispase (2.5 mg/ml) at 4 °C overnight, in order to separate the epidermis from dermis. Epidermal sheets were then put into trypsin-glucose solution (0.25% trypsin; 0.1% glucose) for 30 min at 37 °C, and single epidermal cell suspension was prepared. Cells were counted by improved Neubauer hemocytometer, and then placed at 5×10^5 cells/ml in wells of 96-well plates in culture medium and cultured for 48 h. To examine the effect of warfarin on stimulated epidermal cell cytokine responses, cells were cultured with 100 ng/ml of lypopolysaccharide (LPS).

2.8. MTT assay for cell viability

Epidermal cells viability was determined by a quantitative colorimetric assay described for adherent cells (Oez et al., 1990) which is based on metabolic reduction of tetrazolium salt MTT to a colored end product, formazan. Cells were added to wells of a 96-well plate (1×10^5 epidermal cells/well) and incubated with 500 μg/ml of MTT for 3 h. Formazan produced by the cells was dissolved by overnight incubation in 10% sodium dodecyl sulfate (SDS) – 0.01N HCl and absorbance of extracted chromogen was then measured at 540/650 nm by ELISA 96-well plate reader (GRD, Rome, Italy).