International Journal of Cardiology 168 (2013) 3370–3380

Contents lists available at ScienceDirect

International Journal of Cardiology

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

Local inhibition of ornithine decarboxylase reduces vascular stenosis in a murine model of carotid injury

Amalia Forte [a,d,1,2], Mario Grossi [a,d,e,1,2], Karolina M. Turczynska [e,1], Kaj Svedberg [e,1], Barbara Rinaldi [a,d,1], Maria Donniacuo [a,d,1], Anders Holm [e,1], Bo Baldetorp [f,1], Mariano Vicchio [c,1], Marisa De Feo [b,d,1], Pasquale Santè [b,d,1], Umberto Galderisi [a,d,1], Liberato Berrino [a,d,1], Francesco Rossi [a,d,1], Per Hellstrand [e,1], Bengt-Olof Nilsson [e,1], Marilena Cipollaro [a,d,*,1]

[a] Dept. of Experimental Medicine, Second University of Naples, Italy
[b] Dept. of Cardiothoracic and Respiratory Sciences, Second University of Naples, Italy
[c] Dept. of Anesthesiological, Surgical and Emergency Sciences, Second University of Naples, Italy
[d] Excellence Research Centre for Cardiovascular Diseases, Second University of Naples, Italy
[e] Dept. of Experimental Medical Science, Lund University, Sweden
[f] Dept. of Clinical Science, Lund University, Sweden

ARTICLE INFO

Article history:
Received 16 November 2012
Received in revised form 6 April 2013
Accepted 12 April 2013
Available online 13 May 2013

Keywords:
Restenosis
Negative remodeling
Ornithine decarboxylase
α-Difluoromethylornithine
Endothelial cells
Smooth muscle cells

ABSTRACT

Objectives: Polyamines are organic polycations playing an essential role in cell proliferation and differentiation, as well as in cell contractility, migration and apoptosis. These processes are known to contribute to restenosis, a pathophysiological process often occurring in patients submitted to revascularization procedures. We aimed to test the effect of α-difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase, on vascular cell pathophysiology in vitro and in a rat model of carotid arteriotomy-induced (re)stenosis.
Methods: The effect of DFMO on primary rat smooth muscle cells (SMCs) and mouse microvascular bEnd.3 endothelial cells (ECs) was evaluated through the analysis of DNA synthesis, polyamine concentration, cell viability, cell cycle phase distribution and by RT-PCR targeting cyclins and genes belonging to the polyamine pathway. The effect of DFMO was then evaluated in arteriotomy-injured rat carotids through the analysis of cell proliferation and apoptosis, RT-PCR and immunohistochemical analysis of differential gene expression.
Results: DFMO showed a differential effect on SMCs and on ECs, with a marked, sustained anti-proliferative effect of DFMO at 3 and 8 days of treatment on SMCs and a less pronounced, late effect on bEnd.3 ECs at 8 days of DFMO treatment. DFMO applied perivascularly in pluronic gel at arteriotomy site reduced subsequent cell proliferation and preserved smooth muscle differentiation without affecting the endothelial coverage. Lumen area in DFMO-treated carotids was 49% greater than in control arteries 4 weeks after injury.
Conclusions: Our data support the key role of polyamines in restenosis and suggest a novel therapeutic approach for this pathophysiological process.

1. Introduction

The polyamines spermidine and spermine, and their precursor putrescine, are positively charged molecules expressed by all living organisms and known to play an essential role in cell proliferation and differentiation. They are also involved in cell contractility and migration as well as in apoptosis. All these processes are known to contribute to restenosis after revascularization procedures, a pathophysiological process that is reduced but not eliminated by the advent of bare metal stents and of drug-eluting stents [1].

Several studies have highlighted the multiplicity of mechanisms and levels of action of polyamines in influencing cell functions in healthy and pathological settings. For example, polyamines can modulate the functions of DNA, nucleotide triphosphates and proteins. In particular, polyamines can stabilize the double-stranded DNA by increasing the melting temperature, and can promote DNA bending, thus influencing the recognition of regulatory proteins by their response elements and contributing to regulation of gene transcription [2]. Polyamines can also modulate the functions of RNA and most of them exist in a polyamine-RNA complex in cells [3], revealing their ability to regulate gene expression also post-transcriptionally. Interestingly, polyamines seem to play

* Corresponding author at: Department of Experimental Medicine, Second University of Naples, Via L. De Crecchio, 7-80138 Naples, Italy. Tel.: +39 081 5665930; fax: +39 081 5667547.
E-mail address: marilena.cipollaro@unina2.it (M. Cipollaro).
[1] This author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.
[2] Contributed equally to this study.

0167-5273/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ijcard.2013.04.153

a role also in epithelial-to-mesenchymal transition (EMT), a phenomenon linked to cell plasticity that could have a pivotal function in tissue remodeling [4].

Considering the multiplicity of phenomena in which polyamines are involved, they could play a key role in restenosis progression, as well as to show a potential as therapeutic targets to prevent or reduce this recurrent pathophysiological phenomenon. Indirect evidence for a role of polyamines in stenosis progression has come from preclinical models of vascular injury [5–8]. These findings further supported a role for growth- and migration-stimulatory polyamines in stenosis progression. Despite this premise, the experimental inhibition of polyamine synthesis and/or uptake has been poorly investigated in animal models of vascular disease, as only a few studies so far have focused on the interference with polyamine biosynthesis in restenosis [9,10].

In this study we aimed to analyze the potential therapeutic role of a local perivascular application of α-difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (ODC), in a model of surgically-induced rat carotid (re)stenosis. ODC is the first rate-limiting enzyme in the polyamine anabolic pathway, catalyzing the conversion of ornithine into putrescine, and as such playing a key role in polyamine biosynthesis [3].

The in vivo experiments have been supported and integrated by a panel of in vitro assays performed on rat primary carotid smooth muscle cells (SMCs) and on mouse bEnd.3 endothelial cells (ECs) to examine the influence of DFMO on cell physiology, including possible tissue-specific differences.

2. Methods

2.1. Cells and cell culture

The mouse microvascular EC line bEnd.3 was purchased from American Type Tissue Culture Collection (Manassas, VA, USA). Primary rat carotid SMCs were isolated from male Sprague Dawley rats (250–300 g) euthanized by CO_2 as approved by the regional Animal Ethics Committee, Lund and Malmö. Left and right common carotid arteries were removed and dissected free of surrounding tissue under sterile conditions. Subsequently the vessels were incubated for 30 min at 37 °C in serum free Dulbeccos Modified Eagle's Medium (DMEM) containing 1 mg/mL collagenase type 2 (Worthington Biochemical Corporation, Lakewood, NJ, USA). The adventitia was then pulled off using forceps and the carotid arteries were incubated for 3–4 h at 37 °C in serum free DMEM cell culture media containing 2 mg/mL collagenase type 2 and 0.1 mg/mL elastase (Sigma). The primary SMCs were seeded, allowed to divide and trypsinized (0.25%) upon reaching confluence. The primary rat carotid SMCs were used for experiments in passages 2–4. The bEnd.3 ECs and primary rat carotid SMCs were cultured in a mixture (1:1) of DMEM and Ham's F12 medium with addition of antibiotics (50 U/mL penicillin and 50 μg/mL streptomycin) and 10% fetal calf serum.

All experiments were performed in sub-confluent (~70% confluence) cells. Prior to experiments the cells were made quiescent by culturing with serum-free media for 24 h. The cells were pre-incubated with DFMO for 2 h before they were growth-stimulated with 5% fetal calf serum for 3 and 8 days in the continued presence or absence of DFMO. DFMO was kindly provided by Hoechst Marion Roussel, Cincinnati, OH, USA and dissolved in phosphate buffered saline (PBS).

2.2. Determination of polyamine concentration

The polyamines were determined in cellular homogenate by HPLC and normalized to total protein as described by Seiler and Knodgen [11]. Cellular polyamine concentration was expressed as nmol/mg total protein.

2.3. Immunocytochemistry

Cultured cells were grown on glass cover slips for immunocytochemistry. Cells were fixed in 4% paraformaldehyde for 10 min at room temperature, washed carefully in PBS and then mounted on microscope slides. SM α-actin immunoreactivity was determined using a mouse monoclonal antibody (clone 1-A4, Sigma) at 1:200 dilution. SM22α immunoreactivity was assessed using a rabbit polyclonal antibody (Abcam, Cambridge, UK) at 1:200 dilution. Microtubule structure was assessed using a mouse monoclonal α-tubulin antibody (clone B-5-1-2, Sigma) at 1:4000 dilution. Immunofluorescence was visualized using a Cy3-conjugated secondary anti-mouse or a Cy5-conjugated secondary anti-rabbit antibody at 1:500 dilution (Invitrogen, Carlsbad, CA, USA). The glass cover slips with cells stained for the respective primary and secondary antibody were carefully washed. The nuclei were counterstained with Sytox green (Invitrogen). Fluorescence was analyzed using laser-scanning confocal microscopy (LSM 5 Pascal, Carl Zeiss AG, Göttingen, Germany).

2.4. Determination of DNA synthesis

DNA synthesis was determined by measuring incorporation of radio-labeled methyl-[^{3}H]-thymidine (PerkinElmer Inc., Boston, MA, USA) into newly synthesized DNA. The isotope (5 μCi) was included for the last hour of the 3–8 days incubation with DFMO. The cells were washed in PBS, harvested using a rubber policeman, and sonicated in 5 mM NaOH twice for 10 s. Aliquots of the sonicate were precipitated with 5% trichloroacetic acid and centrifuged (10,000 *g* for 2 min at 4 °C). After washing with 5% trichloroacetic acid, the pellet was dissolved in Soluene. Liquid scintillation cocktail was added and the radioactivity measured in a liquid scintillation counter (Beckman, Fullerton, CA, USA). Radioactivity was expressed as disintegrations per minute (D.P.M.) and normalized to total protein concentration in each sample, determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) based on the Lowry method [12].

2.5. Cell counting and cell viability

The number of cells was determined by counting the trypsinized (0.25% trypsin) cells in a Bürker chamber. The trypan blue exclusion test was used to analyze cell viability. Cell culture medium was removed, cells were washed with 0.9% NaCl and then cells were incubated for 2 min with 0.4% trypan blue (Sigma). Thereafter, the cells were washed 3 times in 0.9% NaCl. The number of cells containing trypan blue was determined as a measure of dead/dying cells.

2.6. Determination of cell cycle phase distribution by flow cytometry

Distribution of cells in the cell cycle was analyzed by flow cytometry of propidium iodide-stained nuclei at 3 days in DFMO treated and control cells as described by Odenlund et al. [13]. Briefly, flow cytometric DNA analysis was performed in a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems; San José; CA, USA). DNA contents were analyzed in about 13,000 nuclei in each sample. Cell cycle phase distribution was determined using ModFit LT 3:1 (Verity Software House Inc., Topsham, ME, USA).

2.7. Animals

Studies were carried out on 12 week-old male Wistar rats (230–250 g) (Charles River, France). Rats were maintained in accordance with the guidelines of the NIH (Guide for the Care and Use of Laboratory Animals, 1976). All protocols were approved by the Animal Care and Use Committee of the Second University of Naples. Rats were acclimatized and quarantined for at least one week before undergoing surgery.

2.8. Vascular injury and DFMO treatment

Rats were anesthetized with i.p. injection of 100 mg/kg ketamine and 0.25 mg/kg medetomidine and carefully placed onto a warm surface and positioned for surgery. All the surgical procedures were conducted with sterile techniques and vital signs were continuously monitored through a pulsioxymeter. Arteriotomy of rat common carotid artery was performed as already published [14]. Briefly, a plastic Scanlom clamp for coronary artery grafting was placed for 10 s on the carotid causing a crushing lesion to the vessel. At the same point where the clamp was applied, a 0.5 mm longitudinal incision was done on the full thickness of the carotid. The incision did not cross to the other side of the vessel. Hemostasis was obtained with a single adventitial 8.0-gauge polypropylene stitch. Once bleeding stopped, the carotid artery was carefully examined and blood pulsation was checked distally to the incision. Rats were administered with 100 μL of 5 mg/mL DFMO in 20% pluronic gel F127 (Sigma) perivascularly applied to the arteriotomy-injured carotid. Control rats were administered with 100 μL of 20% pluronic gel only. Pluronic poloxamer gel is hydrofilic and is liquid at 4 °C but rapidly solidifies when in contact with tissues at 37 °C. After gelification, the skin was approximated by a reabsorbable suture. Animals were allowed to wake up through an intramuscular injection of 1 mg/kg atipamezol. Postoperative systemic analgesia was administered through subcutaneous injection of 0.1 mg/kg buprenorphine every 8 h. Antibiotic therapy was administered through subcutaneous injection of 5 mg/kg enrofloxacin once a day for 3 days following the arteriotomy procedure.

2.9. RNA extraction and RT-PCR analysis

Total RNA was extracted from injured rat carotids at 3 h and at 3 days after arteriotomy ($n = 5$ DFMO-treated rats and $n = 5$ pluronic gel-treated rats, for each time point), from uninjured rat carotids ($n = 4$) and from primary rat SMCs and mouse bEnd.3 cells ($n = 3$ for each group, for each time point) using the RNAeasy minikit (Qiagen) according to the manufacturer's instructions. RT-PCR experiments were performed as already published [15]. GeneBank sequences for rat mRNAs and the Primer Express software (Applied Biosystem) were used to design primer pairs for the genes belonging to the polyamine metabolism and to the other pathways we selected as of interest in our model. Primer sequences are reported in Supplemental Table 1.