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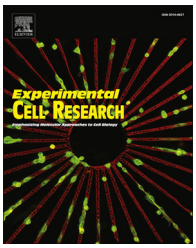

Research Article

12S-Lipoxygenase is necessary for human vascular smooth muscle cell survival

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ABSTRACT

Considerable evidence has been published demonstrating the importance of lipoxygenase enzymes for vascular smooth muscle cell (VSMC) growth. The current study sets out to determine whether or not 12-lipoxygenase (12LO) is also important for human placental VSMC survival. Both a pharmacological and two 12LO antisense knockdown approaches were applied. The 12LO inhibitor baicalien induced a 2–2.5-fold increase in cell death, which appeared to result from apoptosis, as indicated by DNA fragmentation, activation of procaspase 3 to caspase 3 and cytochrome C release from the mitochondria to the cytosol. This apoptosis could be prevented by treatment with the 12LO product, 12 hydroxyeicosatetraenoic acid (12HETE). Human platelet-type 12LO-antisense knockdown, by either plasmid transfection or adeno-associated virus (AAV) infection also induced substantial VSMC death over controls, which could also be prevented by treatment with 12HETE, but not 5HETE. Hence, biochemical 12LO inhibition or 12LO-antisense knockdown in VSMC can induce programmed cell death. These observations suggest a previously unrecognized association between human VSMC survivability and 12LO.

Introduction

Abnormal growth and accumulation of vascular smooth muscle cells (VSMCs) within the arterial intima, resulting in intima-media thickening, appears a common step in the pathogenesis of atherosclerosis. Such growth in VSMC results from alteration in the equilibrium between cell growth and apoptosis [1–3].

Attempts to curb VSMC growth by interfering with key elements in intracellular signalling essential for proliferation generally utilized transfection with antisense oligonucleotide targeting genes such as the E2F transcription factor [4], *ras* and Raf kinase [5] or cell cycle regulating genes, such as *c-myb*, *c-myc*, *cdc*-2, *cdk*-2, proliferating cell nuclear antigen [6,7] and retinoblastoma protein [8]. Although these attempts appeared effective, the experimental setting was generally one of rapid and active VSMC proliferation such as seen following intimal injury. Presently, understanding the targeted retardation of established myointimal thickening such as encountered in

Abbreviations: 12LO, 12-lipoxygenase; AII, Angiotensin II; AAV, adeno-associated virus; Baic, baicalein; CAT, chloramphenicol acetyl transferase; Gfp, green fluorescent protein; HETE, hydroxyeicosatetraenoic acid; MOI, modality of infection; Mp, murine platelet; MCS, multiple cloning site; Nlsgfp, nuclear localization signal expressed green fluorescent protein; VSMC, vascular smooth muscle cells; 5H, 5HETE; 12H, 12SHETE; 600A, human platelet-type12LO cDNA expressed in the antisense orientation

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certain forms of hypertension remains a largely unmet challenge.

12-Lipoxygenase (12LO) is a dioxygenase enzyme that incorporates molecular oxygen into unsaturated fatty acids such as arachidonic acid at carbon position 12 [9]. Arachidonate LOs and their products have been found to play an important role in mediating growth factor induced tumor cell proliferation and appear to enhance the growth and migration of VSMC [10]. As VSMC proliferation is essential in vascular wall function as well as plaque formation and that the inhibition of the renin angiotensin system can induce VSMC apoptosis [11–13] we were prompted in the current study to explore the role of 12 lipoxygenase (12LO) blockade in inducing apoptosis in these cells.

Materials and methods

Materials

Tissue culture medium, FBS and supplements were purchased from Beit HaEmek Biological Industries, Beit HaEmek, Israel. 5HETE, 12SHETE and 15HETE were purchased from Biomol Research Labs, Exeter, UK. For lipofections Metafectene (Biotex, Frankfurt, Germany) was used. G418 was purchased from Sigma Chemical Co., St Louis Mo. USA. Anti-active caspase-3 (MBL (Watertown, MA, USA)) or anti-active caspase-8 antibodies as well as anti cytochrome C were purchased from Santa Cruz Biotech, Santa Cruz, Ca, USA. Anti-actin (clone C4) antibody was purchased from MP Biomedical, Solon, Ohio, USA. Anti-rabbit IgG, and anti-mouse IgG were purchased from Amersham GE Healthcare, Buchinghamshire, UK.

Cells and culture

VSMC were prepared as previously described [14–16]. Umbilical cords were dissected, in accordance with institutional guidelines and the World Medical Association of Helsinki (2000). VSMC were cultured in medium 199 containing 20% fetal calf serum, penicillin G (100 μg/ml) and glutamine, at 37 °C and 5% CO_2 in a humidified incubator. Cell migration was detected within 10–20 days. Cells were used only at the first passage when expression of smooth muscle actin was clearly demonstrable by immunostaining [14,16].

Plasmid construction

The 5′ *Pst I* murine platelet-type cDNA 12LO (mp12LO) 887 bp fragment, (a kind gift from Prof Colin Funk, Queens University, Canada) was cloned into the *Pst I* site of pUC18 (Gibco BRL Life Technologies; Paisley, UK). Then the resulting 900 bp *HindIII-HincII* restriction fragment (with mp12LO antisense with respect to the *Hind III* site) was cloned into the *Hind III*, *Pvu II* precut, bacterial alkaline phosphatase treated pSV_2CAT plasmid vector [17]. The resultant pSV_2-5'mp12LO-A vector, expresses the 5'mp12LO sequence in the antisense orientation, with respect to the SV40 promoter/enhancer.

The *EcoRI* 605 bp human platelet form (smooth muscle variant [9]) of 12LO cDNA fragment was cloned into the *EcoRI* site of the pcDNA3.1+ (Invitrogen; Paisley, U.K.) vector. The resultant pcDNAh60012LOA vector expresses the 605 bp 12LO sequence in the antisense orientation with respect to the CMV promoter/enhancer. All plasmids were prepared in sufficient quantities in our modified triton lysis protocol [18]. All restriction and DNA modifying enzymes were used according to the manufacturers' standard protocols.

AAV2 vector construction and infection

Adeno-associated virus preparation was based on the AAV Helper-Free System purchased from Stratagene (La Jolla, CA, USA). The materials methods for the production of the AAV2 vectors using the AAV293 packaging cells, were described by the manufacturer (Stratagene). The *EcoRI* 605 bp human platelet form (smooth muscle variant [9]) of 12LO cDNA fragment was cloned into the *EcoRI* site of the pAAV2-MCS polylinker (Stratagene) in the antisense orientation with respect to the CMV promoter/enhancer. Viral yield was determined by serial dilution infections of the packaging cell line (AAV293) using GFP as the reporter (Stratagene). Infections were carried out in 25 cm^2 filter flasks (Greiner Industries) over 2 h with mixing every 30 min and then left for 3 days diluted 1:1 (v:v) with fresh complete medium (Stratagene). All manipulations involving live AAV2 virus particles were done in a BL2/3 facility using the precautions advised by the manufacturer and the current NIH guidelines for working with AAV.

Transfections

For stable calcium phosphate transfections, 50% confluent human VSMC cultures were co-transfected with 15 μg of pSV2-5'mp12Lo-A (murine 12LO) or pSV2-600A (human 12LO) with 3 μg of pMCI neo polyA (Stratagene; San Diego, CA, USA). The DNA calcium phosphate complex was prepared as described [19], and allowed to incubate on the cells for 4–5 h, followed by a 90 s glycerol shock [17,19]. G418 selection (250 μg/ml) was applied to the transfected VSMC, for 3 weeks from the day after the transfection and then removed. For stable Metafectene (Biotex, Frankfurt, Germany) lipofections, 1.5×10^5 VSMC cells were seeded 24 h prior to lipofection into 6 well plates in 20% FCS in M-199 medium without additional antibiotics, as described by the manufacturer. Cells were incubated at 5% CO_2 for the indicated periods. For stable lipofections, 1/5th of the DNA used was pMCI neo poly A (Stratagene, San Diego, CA, USA) and stable G418 selection (250 μg/ml) was applied the day following lipofection for 3 weeks.

Cell culture mitochondrial protein preparation for cytochrome C analysis

PBS washed cultured cells were incubated for 5 min with 40 mM Tris (7.4), 1 mM EDTA and 150 mM NaCl and collected with the aid of a rubber policeman. Cells were centrifuged ($1000 \times g$, 4 °C, 5 min), resuspended in buffer B (5 mM Tris-Cl (7.5), 250 mM sucrose, 1 mM EDTA and for each 50 ml of buffer B, one tablet of Roche "Complete" Proteinase Inhibitors (Mannheim, Germany) was added followed by light homogenization (20 strokes on ice with a glass-glass dounce homogenizer), further incubated on ice for 5 min followed by another 10 strokes of homogenization. This homogenate was microcentrifuged for 10 min, 4 °C, at $1000 \times g$. The supernatant was removed and kept on ice, while the pellet was resuspended in 50 μl of buffer B, kept on ice another 15 min, homogenized with 20 strokes and remicrocentrifuged for 10 min (4 °C at $1000 \times g$). The resulting supernatant was collected with