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Protective role of klotho protein on epithelial cells upon co-culture with activated or senescent monocytes

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ABSTRACT

Monocytes ensure proper functioning and maintenance of epithelial cells, while good condition of monocytes is a key factor of these interactions. Although, it was shown that in some circumstances, a population of altered monocytes may appear, there is no data regarding their effect on epithelial cells. In this study, using direct co-culture model with LPS-activated and Dox-induced senescent THP-1 monocytes, we reported for the first time ROS-induced DNA damage, reduced metabolic activity, proliferation inhibition and cell cycle arrest followed by p16-, p21- and p27-mediated DNA damage response pathways activation, premature senescence and apoptosis induction in HeLa cells. Also, we show that klotho protein possessing anti-aging and anti-inflammatory characteristics reduced cytotoxic and genotoxic events by inhibition of insulin/IGF-IR and downregulation of TRF1 and TRF2 proteins. Therefore, klotho protein could be considered as a protective factor against changes caused by altered monocytes in epithelial cells.

1. Introduction

Monocyte interactions with other cells have fundamental role in many biological processes. In cooperation with other cells of immune system, they initiate response to clear invading pathogens, fight inflammation and restore homeostasis [1]. In addition, monocytes interact with epithelial or endothelial cells and accelerate remodeling, mitogenesis and angiogenesis [2–4]. Their direct impact on proliferation and differentiation of chondrocytes [5], astrocytes [6] and mesenchymal stem cells [7] was also evaluated. Mechanisms underlying these interactions appear to be direct cell-cell contact with subsequent release of soluble mediators and good condition of monocytes as a key factor. However, in certain conditions, such as systemic inflammatory response syndrome, chronic inflammation or treatments involving cytotoxic drugs, a population of altered, uncontrollably activated or prematurely senescent monocytes may appear. Monocyte over-activation is associated with persistent, increased release of both, pro- and anti-inflammatory cytokines [8], while senescent cells are characterized as cell cycle-arrested, metabolically active cells ultimately developing senescence-associated secretory phenotype (SASP) [9]. SASP involves altered secretome profile including several families of soluble and insoluble factors, potentially affecting surrounding cells [10]. Although interactions between monocytes and other cells, in terms of their direct pro-proliferatory impact, adhesion and migration have been widely studied, there is still little known about the effect of altered, over-activated or senescent, monocytes on the other cells.

Klotho was originally characterized as a putative age-suppressing gene. In mice, mutation of *klotho* caused extensive premature-aging phenotype and shortened life span [11], while overexpression of klotho extended life span by 20–30% and rescued aging disorders [12]. *Klotho* gene generates two different transcripts: a full-length single-pass transmembrane protein and a secreted short-form. Transmembrane form can be further proteolytically cleaved to short-form klotho and released into circulation [13]. In humans, the expression of secrete klotho predominate over transmembrane form [14]. The presence of klotho protein was reported in a wide variety of tissues including kidney, parathyroid glands, muscular artery, arterial tree, endocrine and neuronal tissues [15–17], whereas secrete klotho is mostly present in the circulation [18]. Interestingly, recent studies revealed much broader biological functions for the membrane as well as secreted klotho protein in many processes. The membrane form of klotho acts a coreceptor for fibroblast growth factor 23 (FGF23) required for FGF receptor activation, whereas secrete klotho functions as a humoral factor with pleiotropic activities in various intracellular signaling pathways, including insulin/insulin-like growth factor-1 (insulin/IGF-1), protein kinase C, cAMP, p53/p21, transforming growth factor (TGF)-β1 and Wnt [19]. Additionally, the intracellular, but not the secreted, form of klotho interacts with retinoic-acid-inducible gene-I

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(RIG-I) and inhibits expression of IL-6 and IL-8 thus suppressing RIG-I-mediated inflammation [20]. Despite our growing understanding of klotho biology, numerous important questions regarding its properties and functions remain still unanswered.

Therefore, the aim of this study, was to evaluate the effect of activated and senescent monocytes on epithelial cells in co-culture in vitro model. Then, taking into account mentioned pleiotropic klotho characteristics, the role of klotho protein in monocyte-epithelial cells interactions was tested.

2. Materials and methods

2.1. Cell lines and cell culture

Human acute monocytic leukaemia cells, THP-1 (ECACC) and human cervical cancer cells, HeLa (ATCC) were grown at 37 °C in RPMI 1640 supplemented with 2 mM glutamine, 10% heat-inactivated FBS and antibiotic-antimycotic mix solution (100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B) (Sigma) in a humidified atmosphere in the presence of 5% CO_2 until they reached confluence. Typically, cells were passaged by trypsinization (HeLa cells) or direct dilution (THP-1 cells).

2.2. Antibodies

Antibodies used were: anti-klotho (#PA5-21078), anti-p16 (#PA5-16639), anti-p21 (#PA5-701151), anti-p27 (#PA5-13254), anti-p53 (#700439), anti-NF-κB (#PA5-37658), anti-β-actin (#PA1-16889), anti-BrdU (#MA3-071), anti-TRF1 (#MA1-46089), anti-TRF2 (#MA1-41001) (Thermo Scientific), anti-active caspase 3 (#NB100-56113), anti-53BP1 (#NB100-304) (Novus Biologicals), anti-Bcl2 (#sc-7382), anti-IGF-IRβ (#sc-9038) (Santa Cruz). Secondary antibodies: HRP-conjugated were: anti-mouse (#A9044), anti-rabbit (#A0545) (Sigma) and FITC-labelled were anti-rabbit (#31583) (Thermo Scientific) and anti-mouse (#orb16690) (Biorbyt).

2.3. Lipopolysaccharide (LPS) and doxorubicin (Dox) stimulation of THP-1 cells

THP-1 cells were seeded at a density of 1×10^6 cells/ml, activated with 1 μg/ml LPS 055:B5 (Sigma #L2880) for 6 h and the effect on cytokine secretion was evaluated with ELISA method (described below). The second model was 72 h treatment of THP-1 cells at a density of 2×10^5 cells/ml with 0.1 μM Dox (Sigma #D1515) and Dox-induced changes were monitored with one-step growth curve, cell cycle and Western Blot analyses.

2.4. Enzyme-linked immunosorbent assay (ELISA)

After stimulation with LPS, cell culture medium was collected and stored at −80 °C, while THP-1 culture was continued in LPS-free medium. After 48 h medium was again collected and amounts of TNFα, IL-1β, IL-6, IL-10 cytokines were quantified using Human TNFα, Human IL-1β, Human IL-6, Human IL-10 ELISA kits according to the procedures recommended by the supplier (Thermo Scientific). Results are expressed in pg of cytokine per ml of cell culture medium.

2.5. One-step growth curve

THP-1 cells were seeded at a density of 2×10^5 cells/ml and treated for 72 h with 0.1 μM Dox. Then, cell culture medium was discarded and replaced with fresh, Dox-free for another 48 h. Cell numbers were counted every 24 h for up to 120 h.

2.6. Cell cycle analysis

The cell cycle was profiled with Muse Cell Cycle Assay Kit and Muse Cell Analyzer (Merck Millipore) according to manufacturer's instruction and using DNA Cell cycle plugin from ImageJ software on photographs taken after nuclei visualization during immunostaining procedure.

2.7. Western Blotting

Whole cell protein lysates were prepared according to Mytych et al. [21], separated by 10% SDS–PAGE and transferred to PVDF membranes, followed by blocking, incubation with the specific primary antibody and secondary HRP conjugated antibody. The proteins were detected using an enhanced chemiluminescence (ECL) substrate (Thermo Scientific) and the relative protein expression levels were normalized to the levels of β-actin (GelQuantNET software).

2.8. Stable transfection of HeLa cells

Klotho (membrane and secrete) plasmids were gifts from Hal Dietz (Addgene plasmid #17712 and #17713, respectively) [22], while control plasmid was obtained from Evrogen (#FP401). HeLa cells were transfected with plasmids by the Lipofectamine LTX Reagent with Plus Reagent (Invitrogen) following the manufacturer's protocol. Stable clones were established by selection in the culture medium containing 1 mg/ml neomycin from the second day of transfection and for approximately 2 months. HeLa cells transfected with plasmids were designated NC (plasmid control), mKL (membrane klotho) and sKL (secrete klotho), while non-transfected cells as control. Transfection efficiency was confirmed with Western Blot – klotho level in total cell protein and secreted into the media (cell culture medium was collected and concentrated in a Microsep Advance Centrifugal Device 10 K MWCO – 15 min, 15,000 rpm, 4 °C) and immunostaining.

2.9. Immunostaining

Immunostaining protocol was used as reported previously [21] with one modification: in contrast to intracellular staining, extracellulary stained cells were not permeabilized. Cells were incubated with the primary antibody and a secondary antibody conjugated to FITC. Nuclei were stained with Hoechst 33342. Digital images were captured with an InCell Analyzer 2000. Quantitative analysis was conducted using ImageJ software and presented as relative fluorescence units (RFU).

2.10. HeLa/THP-1 co-culture

HeLa cells ($2\times10^3/cm^2$) were seeded 24 h before incubation with monocytes. Then, control THP-1 cells (Mo) or stimulated with LPS (Mo LPS) or Dox (Mo Dox) were washed twice with PBS, counted and added to HeLa cells (control, NC, mKL and sKL) at a 2×10^5 cells/ml density to start co-culture experiments. After 48 h, medium with or without THP-1 cells was removed, HeLa cells were washed twice with PBS and fresh medium with or without THP-1 cells was added for another 48 h, for total 96 h of co-culture.

After 96 h incubation, cell culture medium was removed and HeLa cells were used in further analyses.

2.11. Mitochondrial activity – MTT assay

After 96 h co-culture, cell culture medium was removed and replaced with medium containing MTT (500 μg/ml). After 4 h incubation at 37 °C, the medium was discarded, crystals were dissolved in DMSO and the absorbance was detected at 595 nm and at 655 nm (measurement and reference wavelength, respectively) using Tecan Infinite 2000. The readings for the untreated control cells were