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Lactoferrin inhibits neutrophil apoptosis via blockade of proximal apoptotic signaling events

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ABSTRACT

Neutrophils are the most abundant leukocyte and have a short lifespan, dying by apoptosis approximately five days after leaving the bone marrow. Their apoptosis can be delayed at sites of inflammation to extend their functional lifespan, but inappropriate inhibition of apoptosis contributes to chronic inflammatory disease. Levels of the physiological iron chelator lactoferrin are raised at sites of inflammation and we have shown previously that iron-unsaturated lactoferrin inhibited human neutrophil apoptosis, but the mechanisms involved were not determined. Here we report that the anti-apoptotic effect of lactoferrin is dependent upon its iron saturation status as iron-saturated lactoferrin did not affect neutrophil apoptosis. We also show that the effect of lactoferrin is mediated at an early stage in apoptosis as it inhibited activation of sphingomyelinase, generation of ceramide, activation of caspase 8 and Bax and cleavage of Bid. Lactoferrin did not inhibit apoptosis induced by exogenous ceramide, supporting the proposal that it acts upstream of ceramide generation. We therefore conclude that raised lactoferrin levels are likely to contribute to chronic inflammation by delaying neutrophil apoptosis and that this is achieved by inhibiting proximal apoptotic signaling events.

1. Introduction

Neutrophils are the shortest lived cell in the body, surviving for approximately 5 days in the circulation before dying spontaneously by apoptosis [1,2]. Neutrophil apoptosis can be inhibited at sites of infection in order to extend neutrophil bactericidal function [3], but inappropriate extension of neutrophil survival can lead to chronic inflammatory disease [4,5]. Improved understanding of the regulation of neutrophil apoptosis *in vivo* may reveal novel therapeutic targets.

Reactive oxygen species (ROS) have been identified as primary effectors of neutrophil spontaneous apoptosis [6–8] and we have proposed a model for the induction of spontaneous neutrophil apoptosis in which ROS mediate activation of sphingomyelinase, generating ceramide at the cell membrane and inducing clustering and activation of death receptors in lipid rafts leading to activation of caspase 8 with subsequent cleavage of Bid and induction of the mitochondrial death pathway [8]. If this model is correct then factors that influence ROS generation at sites of inflammation would have the potential to modulate neutrophil apoptosis *in vivo* and influence disease pathogenesis.

Here we have considered the role of physiological iron-binding proteins in regulating neutrophil apoptosis and in particular their mode of action. Trace amounts of "free" iron can catalyse the production of hydroxyl radicals via the Fenton/Haber-Weiss reaction [9] and thus influence neutrophil apoptosis. Lactoferrin is an 80-kDa iron-binding protein that is present in secondary granules in neutrophils. The concentration of lactoferrin in the circulation is normally low, i.e. 2.5 nM-7.5 nM, but at sites of inflammation this can be as high as 2.5 μM [10]. Neutrophils release lactoferrin upon activation and can bind lactoferrin, though a specific receptor has not been characterised [11,12]. We have shown previously that iron-unsaturated apo-lactoferrin was able to inhibit the spontaneous apoptosis of human neutrophils *in vitro* [13], though the mechanism of action was not determined.

2. Materials and Methods

2.1. Isolation and culture of human peripheral blood neutrophils

Neutrophils were isolated from the peripheral blood of healthy human volunteers as previously described [14]. All donors gave written informed consent prior to their participation. The purity of isolated neutrophils was determined by Giemsa staining and light microscopy and was routinely greater than 97%. Neutrophils were cultured in RPMI1640 medium (Life Technologies, Paisley, UK)

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containing 10% heat inactivated fetal calf serum (Sera Laboratories International Ltd, Haywards Heath, UK), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, Poole, UK), in the presence or absence of a range of concentrations of human iron-unsaturated apo-lactoferrin, iron-saturated holo-lactoferrin and iron-unsaturated apo-transferrin and anhydrous ferric chloride, $FeCl_3$ (all from Sigma-Aldrich).

2.2. Measurement of neutrophil apoptosis

Neutrophil apoptosis was determined by two methods, observation of nuclear morphology and assessment of mitochondrial membrane integrity. To determine apoptosis by morphology cytospin preparations (3 min, 500 rpm; Cytospin 2; Shandon, Pittsburgh, PA) were made and differentially stained (Diff-Quick; Gamidor, Didcot, U.K.) and assessed for an apoptotic nuclear morphology [15]. Morphological assessments were confirmed by measurement of mitochondrial permeability transition using uptake and retention of M5,5_,6,6_-tetrachloro1,1_,3,3_-tetraethylbenzimidazolylcarbocyanineiodide, JC-1 (Sigma-Aldrich), measured by flow cytometric analysis, as described previously [8]. JC-1 fluoresces in the red channel when present in the mitochondria but fluoresces in the green channel upon its release in to the cytosol [16].

2.3. Measurement of Ceramide

Neutrophils were washed twice in sterile Tris Buffered Saline (TBS, Sigma-Aldrich) and lipids extracted in chloroform:methanol according to the Bligh and Dyer protocol. Ceramide species were measured by HPLC as described previously [17] and the levels combined to give a value for total ceramide.

2.4. Measurement of Caspase 8 activity

Activation of caspase 8 was measured by assessing cleavage of a fluorescently tagged caspase 8 substrate peptide and release of the fluorochrome AMC (R & D Systems, Abingdon, UK). The amount of protein from each sample was measured using the BCA assay (Perbio Science UK Ltd, Cramlington, UK). Results were expressed as relative fluorescence units (RFU) per 100 μg protein. Caspase 8 activity was blocked by incubation of cells with 10 μM of the tetrapeptide caspase inhibitor IETD-fmk to confirm the specificity of the assay (Calbiochem, Nottingham, UK).

2.5. Measurement of Bax activation

The 6A7 anti-Bax antibody is specific for the active conformation of Bax and can be used to measure the degree of Bax activation within cells [18]. Neutrophils were fixed and permeabilised using PermeaFix™ solution (Ortho Diagnostic Systems Inc, Raritan, NJ, USA) and stained with 5 μg/ml affinity purified mouse anti-human active Bax antibody (6A7; Abcam, Cambridge, UK) or normal mouse IgG1 immunoglobulin fraction (Dako UK Ltd, Ely, UK) as a negative control. Staining was detected using a FITC conjugated goat anti-mouse IgG secondary antibody (Southern biotechnology Inc, Birmingham, Al, USA).

2.6. Measurement of Bid cleavage and Mcl-1 expression by western blotting

Full-length Bid (22 kDa) is cleaved by caspase 8 to generate the 15-kDa fragment (tBid) that promotes mitochondrial membrane permeability transition and release of cytochrome *c*. Mcl-1 is an anti-apoptotic protein that is lost during neutrophil apoptisis. Loss of full length Bid and reduced Mcl-1 protein expression were detected by Western blotting as previously described [8]. Briefly, neutrophils cultured for 0 to 20 hours in the absence or presence of 1.25 μM apo-lactoferrin were spun down and the pellet precipitated with ice-cold 10% trichloroacetic acid and the precipitated proteins spun down at 14000 *g* for 5 minutes at 4 °C. The precipitate was washed 3 times in ice-cold acetone and taken up in SDS-PAGE sample buffer and proteins were separated on 12% SDS-PAGE gels. Antibodies to Bid (Biosource International) and Mcl-1 (Santa Cruz Biotechnology, CA, USA) were used in Western blotting, and blots were developed by enhanced chemiluminescence (ECL; Amersham Pharmacia, Amersham, UK).

2.7. Measurement of sphingomyelinase expression and activity

Neutral sphingomyelinase (NSM) activity was measured using the commercial Amplex® Red Sphingomyelinase assay kit (Invitrogen) according to the manufacturer's instructions. Background fluorescence was corrected by subtracting the values derived from a no sphingomyelinase control and standardised to 100 μg of total protein. Quantitative Real Time PCR (qPCR) was used to analyse neutral and acid sphingomyelinase RNA expression in freshly isolated neutrophils. Quantitative PCR was carried out based on the Assay on Demand protocol (Applied Biosystems, Warrington, UK) using pre-mixed 20x TaqMan probe and primer for either neutral or acid sphingomyelinase. Detection was performed using the Mx3005P® QPCR system (Stratagene, La Jolla, CA, USA); cycling conditions were set to 50 °C for 2 minutes, 95 °C for 10 minutes, 45 cycles at 95 °C of 15 seconds and finally 1 minute at 60 °C. Data was analysed using MxPro™ QPCR software (Stratagene) and the relative quantities of mRNA determined against the β-actin gene.

2.8. Statistical analysis

Data presented here represent a minimum of three separate experiments and where appropriate, data are expressed as mean ± SD. Statistical significance was assessed by Student's *t* test and $p<0.05$ was taken as a significantly different value.

3. Results

3.1. Iron saturation abrogates the survival effect of lactoferrin

We have shown previously that iron-unsaturated apo-lactoferrin could inhibit spontaneous neutrophil apoptosis *in vitro* in a concentration dependent manner [13]. To determine whether the iron saturation status of lactoferrin would influence the ability of lactoferrin to enhance neutrophil survival, we compared the effects of iron-unsaturated apo-lactoferrin with iron-saturated holo-lactoferrin on spontaneous neutrophil apoptosis. As shown in Fig. 1A, iron-unsaturated apo-lactoferrin inhibited neutrophil apoptosis, measured by either JC-1 retention by mitochondria or changes to nuclear morphology, but iron-saturated holo-lactoferrin was not able to inhibit neutrophil apoptosis. In addition inclusion of $FeCl_3$ in the medium abrogated the survival effects of apo-lactoferrin (data not shown). To investigate the possibility that the effects of lactoferrin might also have an extracellular component, we determined whether the iron chelator transferrin could also delay neutrophil apoptosis. Neutrophils do not express the transferrin receptor [18]. As shown in Fig. 1c iron-unsaturated apo-transferrin had no effect on the survival of neutrophils even after culture was extended to 20 h.

3.2. Apo-lactoferrin inhibits activation of sphingomyelinase in neutrophils

We have proposed that neutrophil apoptosis is initiated by loss of redox status and accumulation of ROS leading to activation of sphingomyelinase, resulting in the generation of ceramide and activation of death receptor signaling [8]. However in our previous study we did not consider the differential involvement of acid and