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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

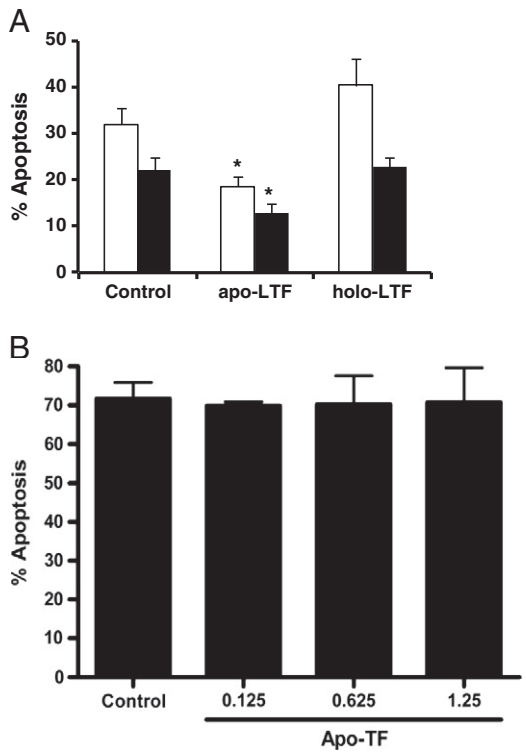

Fig. 1. Iron-unsaturated lactoferrin but not iron-saturated lactoferrin inhibits spontaneous neutrophil apoptosis. A. Human neutrophils were incubated with medium alone or with medium containing 1.25 μM iron-unsaturated apo-lactoferrin (Apo-LTF) or 1.25 μM iron saturated holo-lactoferrin (Holo-LTF). Apoptosis was measured by analysis of nuclear morphology (filled bars) or JC-1 retention by mitochondria (open bars) after 9 h in culture and data are presented as the % of apoptotic cells. B. Neutrophils were incubated with medium alone or with medium containing 0.125 – 1.25 μM iron-unsaturated apo-transferrin (Apo-TF) and apoptosis measured by analysis of nuclear morphology after 20 h in culture. Data are mean ± SD (n = 3) and * indicates $p<0.05$, for treated versus control.

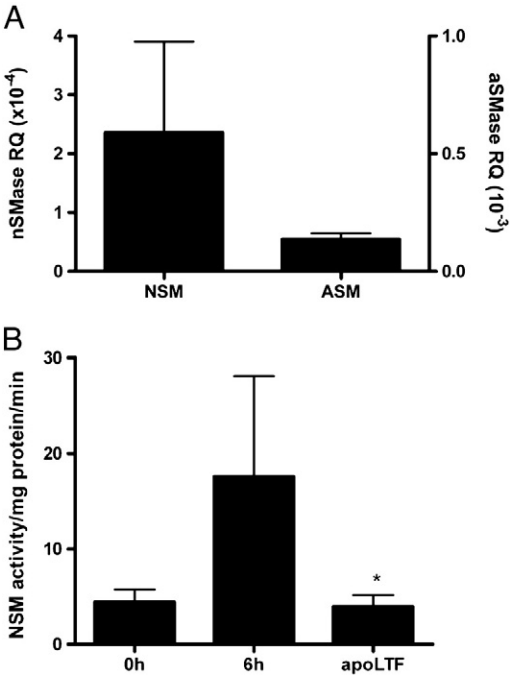

Fig. 2. Apo-lactoferrin inhibits neutral sphingomyelinase activity in neutrophils. A. Expression of ASM and NSM in freshly isolated neutrophils was determined by quantitative PCR. Data are mean ± SD (n = 3). B. NSM enzymatic activity was measured in freshly isolated neutrophils (0 h) and neutrophils cultured in the absence (6 h) or presence (apo-LTF) of 1.25 μM iron-unsaturated apo-lactoferrin for 6 h. The data are expressed as NSM activity per mg protein per minute and are mean ± SD (n = 7).* indicates $p<0.05$.

neutral sphingomyelinases, although neutral sphingomyelinase has been shown to be sensitive to redox status [19]. We therefore measured expression of both acid (ASM) and neutral sphingomyelinase (NSM) by quantitative PCR. Fig. 2A shows that neutrophils expressed both forms of sphingomyelinase, though NSM was the dominant form. However, when we attempted to determine sphingomyelinase activity enzymatically as neutrophils aged in culture, we found a significant increase in NSM with time (Fig. 2B), but we could not reproducibly detect ASM activity. These data suggest that NSM is the major source of ceramide during neutrophil apoptosis. Importantly we showed that iron-unsaturated apo-lactoferrin was able to inhibit NSM activation as neutrophils aged in culture (Fig. 2B).

3.3. Apo-lactoferrin inhibits early signalling events in neutrophil apoptosis

To determine if the inhibition of sphingomyelinase activation by apo-lactoferrin was sufficient to impact upon downstream apoptotic signalling we assessed the effect of apo-lactoferrin upon ceramide generation, caspase 8 activation, Bax activation and Bid cleavage and loss of the major neutrophil anti-apoptotic protein Mcl-1. As shown in Fig. 3A, freshly isolated neutrophils contained minimal amounts of ceramide which increased significantly by 6 hours of culture ($p<0.05$). Increased ceramide levels were not seen in neutrophils treated with apo-lactoferrin (Fig. 3A). In addition, the increase in caspase 8 activity seen during spontaneous neutrophil apoptosis was inhibited by apo-lactoferrin, but not by holo-lactoferrin (Fig. 3B).

Bax activation and insertion into the outer mitochondrial membrane contributes to mitochondrial permeability transition and initiation of the mitochondrial cell death pathway [20]. The conformational change associated with activation can be detected using the 6A7 antibody. Fig. 3C shows that lactoferrin inhibited the Bax activation that occurred during spontaneous neutrophil apoptosis. Bid is cleaved to tBid by caspase 8 and subsequently also inserts in to the mitochondrial membrane [21]. Iron-unsaturated lactoferrin also inhibited the loss of full length Bid during spontaneous neutrophil apoptosis (Fig. 3D). Loss of Mcl-1 is a major driver for spontaneous neutrophil apoptosis [22] and Fig. 3D shows that apo-lactoferrin was able to prevent the down-regulation of this protein as neutrophils aged in culture. Taken together these data suggest that lactoferrin interferes with neutrophil apoptosis at an early stage in the apoptotic process.

3.4. Apo-lactoferrin cannot inhibit apoptosis induced by exogenous ceramide

If lactoferrin is acting upstream of ceramide generation it should not be able to block apoptosis induced by addition of exogenous ceramide. Fig. 4 shows that addition of exogenous ceramide to neutrophils was able to increase neutrophil apoptosis significantly ($p<0.02$) and that this could not be blocked by lactoferrin. These data support the proposal that lactoferrin acts upstream of ceramide generation at the level of sphingomyelinase activation.

4. Discussion

Here we confirm that the physiological iron binding protein lactoferrin was able to inhibit neutrophil spontaneous apoptosis and show that the anti-apoptotic effect was entirely dependent upon its iron saturation status. Previous work has shown that in zymosan stimulated neutrophils iron bound to lactoferrin is 5000 times more potent than FeCl3 in generating hydroxyl radicals. Furthermore iron-unsaturated lactoferrin was able to reduce hydroxyl radical generation close to the concentration range used here [26]. The ability of the

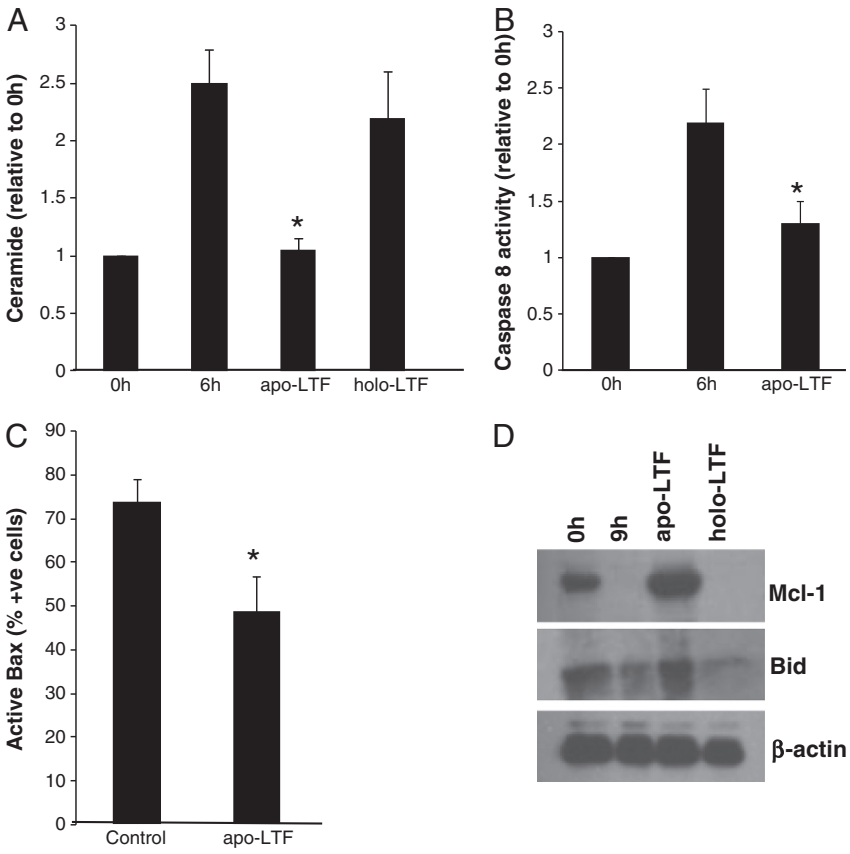

Fig. 3. Apo-lactoferrin inhibits early signalling events in neutrophil apoptosis. Neutrophils were incubated with medium alone (0 h and 6 h) or with medium containing 1.25 μM iron-unsaturated apo-lactoferrin (apo-LTF), 1.25 μM iron-saturated holo-lactoferrin (Holo-LTF) for 6 hours. A. Lipid extracts were prepared and analysed for ceramide content by HPLC. Data are mean ± SD (n = 3) and are expressed relative to those in freshly isolated cells. * indicates $p<0.02$. B. Caspase 8 activity was measured using a fluorometric assay and data are expressed as RFU per μg protein relative to the value for freshly isolated cells (0 h). Data are mean ± SD (n = 4) and *indicates $p<0.05$ compared to the 6 h value. C. Bax activation was measured by immunostaining and flow cytometry using an antibody specific for the active conformation of Bax. Data are expressed as % cells positive for the active confirmation of Bax and are mean ± SD (n = 5), * indicates $p<0.04$. D. Neutrophil proteins were extracted after incubation of cells with medium alone (0 h) and after 9 h in the absence (9 h) or presence (+ apoLTF) of 1.25 μM apo-lactoferrin (apo-LTF) or 1.25 μM holo-lactoferrin (holo-LTF). Bid activation, assessed by the loss of full length 22 kDa Bid, and loss of Mcl-1 were both detected by western blotting. The image shown is representative of three separate experiments.

iron chelator desferrioxamine to inhibit neutrophil apoptosis was also ablated by reduction of its iron chelating ability by addition of $FeCl_2$ [27], adding support to our suggestion that the ability to prevent hydroxyl radical generation via the Fenton reaction is fundamental to the effects of iron chelators on neutrophil apoptosis. Others have reported that generation of hydroxyl radicals is involved in both spontaneous and activation-induced neutrophil apoptosis [7]. Taken together these data suggest that the ability of iron-unsaturated lactoferrin to inhibit neutrophil spontaneous apoptosis is dependent upon its iron saturation status and its effects may be mediated by its ability to reduce hydroxyl radical generation.

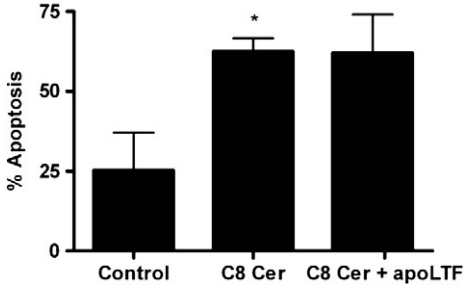

Fig. 4. Apo-lactoferrin does not inhibit apoptosis induced by exogenous ceramide. Neutrophils were incubated for 9 h in medium alone, medium containing 100 ng/ml C8 ceramide or 100 ng/ml C8 ceramide and 1.25 μM iron-unsaturated apo-lactoferrin (apoLTF) and apoptosis determined by DiOC6 staining. Data are mean ± SD (n = 3) and * indicates $p<0.02$.

We have suggested previously that ROS are involved in the initiation of neutrophil spontaneous apoptosis via their ability to activate death receptor signalling in the absence of death receptor ligation [8]. A similar mechanism has been demonstrated for apoptosis induced by chemotherapeutic agents and UV-irradiation [24]. This mode of apoptosis initiation involves the activation of sphingomyelinase, leading to the generation of ceramide-rich lipid rafts, which then induce activation of death receptors such as Fas. In our previous work we showed that neutrophil apoptosis was reduced, but not completely blocked in ASM −/− mice [8], but we did not determine whether ASM was activated during human neutrophil apoptosis. Here we showed that NSM was the dominant sphingomyelinase at the mRNA level, that NSM enzymatic activity increased dramatically as human neutrophils aged and this was inhibited by apo-lactoferrin. In contrast we could not detect ASM activity during spontaneous human neutrophil apoptosis and we therefore propose that although ASM does play a role in early events in neutrophil apoptosis in mice [8], NSM is likely to be the major source of ceramide generated as human neutrophils enter apoptosis spontaneously. Interestingly, a recent report has revealed that both ASM and NSM are redox sensitive, but that they are activated by distinct reactive oxygen species. NSM is activated predominantly by hydroxyl radicals, whereas ASM is activated preferentially by peroxynitrite [25]. Moreover, previous work has shown that generation of hydroxyl radicals is involved in neutrophil apoptosis [7] and apo-lactoferrin has been shown to reduce generation of hydroxyl radicals by neutrophils [26]. Taken together these data add support to our proposal that

hydroxyl radical generation and NSM activation is the target of apo-lactoferrin in regulating neutrophil apoptosis.

Our data also showed that lactoferrin was able to block several downstream events in death receptor signaling in neutrophil apoptosis, such as caspase 8 activation, Bid cleavage and loss of Mcl-1. Crucially, the finding that neutrophil apoptosis could still be induced in the presence of lactoferrin if exogenous ceramide was supplied, supports our suggestion lactoferrin's actions include the blocking of the initial activation of sphingomyelinase. Recently, others have shown that cathepsin D is involved in activation of caspase 8 during neutrophil apoptosis and that the release of cathepsin D from azurophilic granules into the cytosol was ROS dependent [23]. Future studies in our group will investigate whether lactoferrin also inhibits this release of cathepsin D into the cytoplasm. We have also to consider that lactoferrin may provide survival signals via other means and in this context others have shown that iron-unsaturated lactoferrin also increased adherence of neutrophils to endothelial cells [28]. Although not investigated in our study it is possible that such an effect would provide an additional contact-mediated survival signal.

4.1. Conclusions

Iron-unsaturated apo-lactoferrin inhibits spontaneous apoptosis in human neutrophils, a process which is dependent upon the iron saturation status of this agent and involves blockade of the very earliest events in the apoptosis process in these short lived cells.

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