Effects of lactoferrin on the survival biology essay

Science, Biology

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Introduction

In human tissues and organs, a balance in cell number is often maintained by a strict control of cell birth and cell loss [1]. However, occasionally, the balance could be broken by deregulation of cell proliferation and division, which may lead to cancer. The uncontrolled cell growth is associated with several genes, known as oncogenes, which were frequently mutated or highly expressed in tumour cells [2]. Oncogenes are mainly responsible for activating cell proliferation and enhancing tumour survival through secretion of growth factors, cell growth associated receptors, transcription factors and cell proliferation signaling pathways mediated molecules or regulating cell cycles (As reviewed in this paper[3]). Those proteins / molecules secreted by either the tumor cells or their neighbouring cells, are important factors that drive tumour growth. However, tumorigenesis is a complex process involving a variety of mechanisms. Those mechanisms in detail can be studied under a great framework raised by Hanahan and Weinberg, suggesting six hallmarks and two emerging hallmarks of cancer, which include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism and evading immune destruction [4, 5]. Apart from tumour cells, the interaction between cells contributes to the complexity of tumour microenvironment. One chief characteristic of cancer is resisting cell death. Programmed cell death by

apoptosis is thought to be the main fraction of cell loss. Failure of cells to undergo apoptosis will lead to various diseases including cancer. In tumour cells, although the amount of cell death is far less than what arises from cell proliferation and differentiation, apoptotic cells played an important role in regulating the neighbouring tumour cells by interacting with the microenvironment of tumour. Different studies carried out on the molecular mechanism of apoptosis in cancer, has exposed many proteins and molecules that play key roles in regulating the fate of tumour cells. Studies have shown that deregulation of apoptosis by oncogene mutations can contribute to neoplastic transformation, progression and metastasis (As reviewed in this paper [6]). Meanwhile, resisting apoptosis can augment the ability of tumours to escape the surveillance of immune cells [7]. However, recent studies demonstrated some interesting findings, which contradict our current knowledge. Evidence suggests that p53-induced apoptosis triggered by γ-irradiation upon DNA damage can promote tumorigenesis [8, 9]. By knocking out the pro-apoptotic gene PUMA in hematopoietic stem/progenitor cells in mice, they found that mice can resist lymphomagenesis by showing reduced compensatory proliferation, replication and stress-associated DNA damage. CD95, also known as FasR and APO-1, is a death receptor that regulates tissue homeostasis by initiating apoptosis. In human cancers, CD95 is normally downregulated but still expressed on human cell surface [10]. However, recently, CD95 has been shown to be an enhancer of oncogenesis in various cell lines [11]. By knocking down or knocking out CD95, researchers have found that CD95 contributes to tumour growth rather than tumour suppression in different human and primary cancer cell lines. This is

consistent with the results obtained from the studies using mouse models for ovarian and liver cancer, by knocking out CD95, thus reducing cancer incidence and tumour size. These paradoxical findings steer our attention towards the therapeutic strategies that could be designed for cancer treatment. In addition, studies involving the influence of apoptotic cells on tumour formation and growth are becoming more critical in sorting this complex question out. Net tumour growth is achieved by new cell birth outweighing cell loss. Therefore, cell loss outweighing cell birth can be a potential treatment for cancer. Malignancy of tumour is defined by its ability to metastasize to the other parts of the body through blood and the lymph system. In high-grade malignant tumours, high rate of cell proliferation is associated with high rate of apoptosis [12]. Examples of this concept are seen in non-Hodgkin’s lymphoma (NHL), including Burkitt’s lymphoma, immunoblastic lymphoma, pre-B and pre-T lymphoblastic lymphoma and thymoma (As reviewed in this paper [13]). In many aggressive tumours, although there is high rate of cell death, very few apoptotic bodies are observed. This indicates a very active clearance mechanism [14]. This feature of malignant tumours indicates that apoptotic tumour cells might play an important role in regulating tumour growth in various ways. Using histological studies, a large number of macrophages, termed as ‘ starry-sky’ macrophages, have been seen accumulated in Burkitt’s lymphoma, indicating that an active phagocytic clearance of apoptotic tumour cells occurred in those cases. The recruited tumour associated macrophages (TAM) activates anti-inflammatory, immunosuppressive and repair processes and is probably associated with development and growth of tumour [13].

Fractalkine has been shown to drive the peritoneal macrophage-mediated engulfment of apoptotic cells through the activation of MFG-E8 [15]. Later studies show that fractlkine is released by apoptotic lymphocytes to attract macrophages via caspase and Bcl-2 mediated pathways [16]. However, the role of TAM in mediating the microenvironment of tumours is largely unknown. One hypothesis is that apoptotic tumour cells can drive trophic responses by triggering the accumulation of macrophages to produce tumour growth and survival factors [13]. Another trophic effect that is produced by the apoptotic tumour cells is the activation of compensatory proliferation in the neighbourhood, but the underlying mechanism is unclear. Apoptotic cells might also produce immunosuppressive signals that inhibit host immune responses [13]. Recently, it has been found that apoptotic cells produce lactoferrin (LF) [17]. In the past 25 years, with the advent of achievements in human health, various studies involving the function of LF has been carried out, but more research to investigate the effect of LF in cancer need to be done [18]. Most studies suggest that LF inhibits cell proliferation and suppresses tumour growth both in vitro and in vivo [19, 20, 21, 22]. In human breast cancer, studies demonstrated that treatment of MDA-MB-231 cells with LF results in growth arrest at the G1 to S transition of the cell cycle [19]. Another angiogenesis inhibiting effect has been found in vivo model by orally administrating bovine LF to rats [20]. Researchers have found that LF treatment significantly inhibit VEGF165-mediated angiogenesis, which highlights an inhibition for one hallmark of cancer. However, restricted by the heterology between bovine LF and human LF, this work has lesser applications. Looking into head and neck district, human and

murine recombinant LF have been injected into murine models to test the effect on squamous cell carcinoma and fibrosarcoma. A significant effect in reducing tumour volume was demonstrated in both types of tumours in the immunocompetent model while no significant difference was seen in a nude-mouse model [22]. These experiments indicate that LF suppresses carcinoma and fibrosarcoma growth by activating immune responses. However, LF in different district within our body in fighting tumours might differ because of the different tumour microenvironment. LF is an 80-kDa iron-binding glycoprotein, found in milk, saliva, tears, semen and mucosa [23]. Apart from its exocrine secretions, it showed a high concentration in granules of neutrophils [24]. Its iron binding property has been shown associated with various functions such as anti-microbial, anti-viral and anti-inflammatory. Its anti-microbial ability was first found to sequester iron by binding to Fe3+, providing a low level of iron for microbial growth [25]. The iron-independent mechanism via direct interacting with microbial surface components was later demonstrated [25]. In addition to its anti-microbial ability, the ability of LF in protecting infection and septic shock has been demonstrated in many studies in vivo and in vitro [26]. Its anti-inflammatory ability mainly lies on downregulating pro-inflammatory cytokines (TNF-, IL-1, IL-6) and enhancing the anti-inflammatory cytokines (IL-4, IL-10) [26]. In addition, LF has an effect on innate and adaptive immune cells by enhancing the recruitment of neutrophils and macrophages and activation of dendritic cells [27]. Also, LF is a growth factor for osteoblasts through its binding to low-density lipoprotein receptor-related protein 1 and increased COX-2 and NFATc1 activities, indicating a role of mediating mitogenic effects in

osteoblasts [28, 29]. Furthermore, LF has been found as a survival factor in rheumatoid synovial fluid by delaying the apoptosis of neutrophils [30]. In early stage of apoptosis, LF delayed the apoptosis via blockade of proximal apoptotic signaling events [31]. Recently, unexpected evidence showed that LF generated by apoptotic human cells of diverse lineages, including tumour cells, has the ability to inhibit migration of neutrophils [17]. Neutrophils are always absent in tumour growth. By inhibiting the migration of neutrophils, LF might works as a pro-tumour factor in this respect. Thus, lactoferrin released by apoptotic tumour cells has the potential to promote tumour growth through various mechanisms: prevention of granulocyte infiltration, direct trophic effects on tumour cells and indirect trophic and immunomodulatory effects via activation of TAM [13]. Here, taking the anti-inflammatory ability of LF, pro-growth effects on osteoblasts and survival effects on neutrophils into account, we hypothesized that LF acts as a survival and growth factor of lymphoma cells. Our aim is to test whether human LF can delay apoptosis of lymphoma cells or promote lymphoma cells growth in vitro.

Materials & Methods

Reagents

Human milk derived lactoferrin was purchased from Sigma-Aldrich (Purity: ≥85%), recombinant lactoferrin and bovine lactoferrin were purchased from Sigma-Aldrich (Purity: ≥90%) as well. Annexin V binding buffer was made of 140mM NaCl (Fisher Scientific), 10mM HEPES (Sigma-Aldrich), 2. 5mM $CaCl_2$ (Sigma-Aldrich).

Cell culture

The Burkitt's lymphoma cell lines BL2 and MUTU I were cultured in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (PAA laboratories Ltd, Yeovil, UK), 100U/ml penicillin (PAA Laboratories), 100ug/ml Streptomycin (PAA Laboratories) , 2mM L-Glutamine (PAA Laboratories) , at 37℃ under an atmosphere of 5%CO2. BL2 cells were also maintained in 50% RPMI supplemented with 50% x-vivo 20 (Lonza, Slough, UK), 100U/ml penicillin (PAA Laboratories), 100ug/ml Streptomycin (PAA Laboratories) and 2mM L-Glutamine (PAA Laboratories).

Cell Counting

The growth of BL2 cells in complete media or 50% x-vivo media was measured by cell counting. Bl2 cells were taken from complete media or 50%x-vivo media, diluted into three densities (0. 1x105/ml, 0. 2x105/ml, 0. 4x105/ml), and seeded in a 24 wells plate (1ml) for each density with duplicates on day 1. Cells were stained with trypan blue (Sigma-Aldrich) and counted under a microscope (Carl Zeiss Ltd) with a hemocytometer (Hawksley) for the next 4 days.

Induction of apoptosis by nutrient deprivation, chemicals or radiation

BL2 Cells were taken from 50%x-vivo media, MUTU I cells were taken from complete media, washed twice with Dulbecco's PBS (PAA) at 300g for 5mins, resuspended in 0. 22μM filtered RPMI1640 supplemented with 0. 5% bovine serum albumin (low endotoxin; Sigma-Aldrich) at a density of 1x106/ml (Unless otherwise stated). Cells were seeded in a 48 wells plate (0. 5ml) and a broad range of human lactoferrin, recombinant lactoferrin or bovine

lactoferrin were added as specified followed by an incubation for 24h or 48h. 1μM staurosporine (Calbiochem, San Diego, CA, USA) or ultraviolent B (UVB) radiation were also used as stimuli to induce apoptosis. Cells were treated in same way as described above for set up, lactoferrin were added together with 1μM staurosporine or before UVB treatment, and then incubated for a certain time as specified. After incubation, cells were rinsed once with cold PBS at 300g for 5mins. 100ul Annexin V binding buffer were used to resuspend cells and 1ul Alexa Fluor-488 conjugated Annexin V (Invitrogen, Paisley, UK) were added followed by an incubation for 15 mins. 10ul 20ug/ml propidium iodide (Invitrogen) was added to stain cells before analysis by flow cytometry. Levels of apoptosis were measured by flow cytometry (Beckman Coulter, High Wycombe, UK) as described below.

Analysis of cell viability

Cells were stained with Annexin V/PI to assess early or late apoptosis by flow cytometry. Cells that stained Annexin V/PI double negative were considered alive, cells that only stained with Annexin V were defined as apoptotic cells, cells that stained with PI only or double positive were considered to be dead cells.

Fluorescence microscope

The viability of cells in nutrient deprived condition was also visualized by fluorescence microscope. 1 ml BL2 cells (1x106/ml) were collected from serum free media supplemented with bovine serum albumin at 0h and 24h followed by a wash with 2ml PBS. 1% formaldehyde (Fisher Scientific) was diluted and added to fix the cells (at least 30 mins). After fixing, cells were

suspended down and stained with 50ul 500ng/ml DAPI (Invitrogen) in dark for 2 hours. Slides were made with 10ul solution and the viability of cells was observed using a fluorescence microscope (Carl Zeiss Ltd). Images were taken using a LeicaDFC500 camera.

Results

BL2 cells grew well in 50% x-vivo media while reach to plateau phase with a lower density compared to cells grew in complete media

To test the effects of LF on Burkitt's lymphoma in vitro, BL2 cells have been chosen as a start model because of the high rate of spontaneous apoptosis property. Bovine lactoferrin is in the fetal bovine serum (FBS), therefore 50% x-vivo media was used to culture BL2 cells for the sake of serum depletion. Trypan blue is a diazo dye which cannot pass through an intact membrane of live cells but can stain dead cells with a blue colour, therefore it was used to distinguish dead cells from live cells. The growth of BL2 cells was displayed by growth curves in media with or without serum (Figure 1). In complete media, cells can grow to as high as 2. 5x106/ml and entered the plateau phase afterwards (Figure 1A and 1B). By contrast, cells in 50% x-vivo media grew slower and entered the plateau phase with a relatively lower concentration (about 1. 4x106/ml) (Figure 1C and 1D). It might because cells grew in serum free media is more fragile when less growth factors is provided. It also suggests that a lot of growth or survival factors were in the serum to help cells grow but not compulsory. In complete media or 50% x-vivo media, the highest seeding concentration (4x105/ml) of cells have a shorter lag phase or log phase and entered into the plateau phase on day 4

while the cells with a lower seeding concentration (2x105/ml or 1x105/ml) were still in log phase. From these growth curves, experiments were set up with BL2 cells taken from log phase in 50% x-vivo media, with a strict constraint that setting up each experiment on day 2 or day 3 after splitting in a density of 2x105/ml or 1x105/ml, respectively. ABCDFigure 1 BL2 cells growth curve in complete media or 50% x-vivo media. Cells were seeded at 4x105, 2x105, 1x105cells/ml in complete media or 50% x-vivo media, grown for 5 days and counted each day with trypan blue. (A) Trypan blue negative cells (white cells) counts in complete media (n= 4). (B) Total cells counts in complete media (n= 4) (C) Trypan blue negative cells (white cells) cells counts in 50% x-vivo media (n= 3). (D) Total cells counts in 50% x-vivo media (n= 3).

Human lactoferrin showed a death promoting effect in nutrient deprived condition but not reproduciable with a new sample

To examine the effects of LF on the survival of BL2 cells, different ways were used to induce cells undergo apoptosis. Whether LF can delay apoptosis or prevent cells to die was assessed by Annexin V/ propidium iodide (AxV/PI) staining. Annexin V is a protein with a high binding affinity to phosphatidylserine (PS) while PI is a fluorescence molecule which binds to nucleic acids. BL2 cells were exposed to nutrient deprived condition for 48 hours and AxV /PI staining was carried out to check the viability of cells in 24h and 48h using flow cytometry (Figure A). Dot plots of BL2 cells (Figure Ai) showed about 60% BL2 cells were successfully triggered to death in nutrient deprived condition within 48h. About the same proportion of BL2

cells was confirmed dead shown in the AxV-/PI- region from 0h to 48h. However, the proportion of apoptotic cells (AxV+/PI-) is only about 10% at 24h (Figure Aii). By adding human milk derived lactoferrin (hLF) ranging from 0. 01ug/ml to 1ug/ml does not show any significant effect on the survival of BL2 cells under nutrient deprived condition at both 24h and 48h (Figure Aiii). Cell death through apoptosis in nutrient deprived condition was further confirmed by DAPI staining. DAPI is used as a DNA stain which can pass through the intact membrane though stain less effectively in live cells. Nuclei undergo fragmentation during apoptosis and more apoptotic cells have been seen in 24h compared to 0h (Figure B). Another method is to expose cells to a cell kinase inhibitor staurosporine (STS) for 1h or 3h to investigate the effects of human LF (Figure C). The majority of cells were in the viable gate with an exposure to STS for 1h while most cells were shifting to non-viable gate with an exposure to STS for 3h (Figure Ci). By adding hLF ranging from 0. 01 to 1ug/ml, however, the percentage of cells undergoing apoptosis did not differ between the control group and cells incubate with hLF with STS treatment for 1h and 3h. Perhaps the concentration of hLF is too low to generate enough effects on the survival of cells. AiSide scatter (SS)0200400600800100002004006008001000 9. 8889. 2020040060080010000200400600800100023. 273. 3020040060080010000200400600800100066. 228UT 0hUT 24hUT 48hForward scatter (FS)AiiAnnexin V (AxV)Propidium iodide(PI)10-110010110210310-11001011021030. 99. 172. 52nd incubated vation, nt LF were added but still make no difference between groups. nsity compared to 87. 410-110010110210310-11001011021030. 6620. 611. 267. 510-

110010110210310-11001011021031. 7164. 111. 223UT 0hUT 24hUT48hAiiiB0h 24h2013. 02. 12 BL2 0h DAPI 40x 3. tif2013. 02. 12 BL2 24h DAPI 40x 0004. tifCiSide scatter (SS)Forward scatter (FS)0200400600800100002004006008001000110011. 686STS 1h0200400600800100002004006008001000690069. 727. 6STS 3hCiiFigure 2 Testing effects of hLF on the survival of BL2 cells by using different ways to induce apoptosis. Apoptosis levels were assessed by flow cytometry following staining with AxV/PI. (A) BL2 cells were taken from 50% x-vivo media and grown in RPMI serum free media (RSF) supplemented with bovine serum albumin(BSA) for 48h, human LF were added from the incubation with RSF (n= 2). (i) Light Scatter profile of untreated group(UT) at 0h, 24h and 48h. (ii) Cells state of UT group at 0h, 24h and 48h. (iii) The viability of cells in nutrient deprived condition incubated with or without human LF. (B) Cells in nutrient deprived condition were stained with DAPI at 0h and 24h. Bright blue dots suggest the strong binding with DNA of the apoptotic or dead cells. Arrows indicate the fragmented DNA in the late stage of apoptosis. Original magnification, x400. (C) BL2 cells were treated with 1μM STS for 1h and 3h incubated with or without hLF (n= 2) (i) Light profile of cells with an exposure to STS at 1h and 3h. (ii) The viability of cells in the presence of STS incubated with or without hLF. To test this, the dose of LF was increased and recombinant LF (recLF) was added for testing. Two methods that used before were performed to induce apoptosis, additionally with an introduction of ultraviolet B (UVB) exposure. BL2 Cells in nutrient deprived media alone for 2h suggests a small proportion of cells (about 20%) were successfully undergo apoptosis within 2h but no significant effect have been seen in the

presence of a range (5, 10, 20ug/ml) of hLF or recLF (Figure 3A). About 60% cells (Red bar) were undergo apoptosis with 1μM STS treatment at 2h. By adding hLF or recLF, no significant difference have been shown in this case (Figure 3B). A further incubation for 140mins after an exposure to UVB for 15mins have triggered a death rate of 60% (Blue bar). Human LF and recombinant LF were added but still make no difference between groups (Figure 3C). It might because LF do not have enough time to act upon these short term stimuli, e. g. STS, UVB. ABCFigure 3 Testing effects of human LF and recombinant LF with an increased concentration on BL2 cells survival using nutrient deprivation, STS or UVB treatment. BL2 cells were taken from 50% x-vivo media and grew them in RSF supplemented with BSA, human or recombinant LF were added from the incubation with RSF. Apoptosis levels were assessed by flow cytometry following staining with AxV/PI. (A) Cells grew in RSF for 2h with or without LF (n= 1) . (B) Cells were treated with 1μM STS and incubated with or without LF for 2h (n= 2) . (C) Cells were treated under UVB (100mJ/cm2) for 15mins and incubated with or without LF for 140mins (n= 1) . The nutrient deprivation experiement was performed to trigger cells undergo apoptosis. Taking the interation of mutual survival effects of cells in culture into account, BL2 cells were seeded at three different concentrations and hLF (10ug/ml) was added as a test. After 24h incubation, cells seeded at a lower density without hLF showed a poorly survival of cells (Blue bar) compared to cells seeded at a higher density. Intriguingly, by adding hLF, the viability of BL2 cells (Blue bar) with a seeding concentration of 0. 5 x106/ml or 1x106/ml, dropped more than half from about 32% to 12% or 58% to 23%, respectively (Figure 4A). In a dose

dependent experiment, hLF begin to show a significant cell death promoting effect at a concentration of 2. 5ug/ml(Figure 4B). However, does dependent cell death promoting effects of hLF have not been seen with an increase of concentration from 2. 5ug/ml to 20ug/ml. Interestingly, recombinant and bovine LF(10ug/ml) does not show any effect on the survival of BL2 cells. It might suggusts that recLF and bLF acts differently from hLF towards cells. From this experiment, because the effect of hLF is dose independent, we suspect that the cell death promoting effect is whether the effect of hLF itself or not. To confirm our obervation, a new preperation human LF sample was tested. Unfortunately, the cell death promoting effect did not shown when using the new sample while the effect of old sample persists (Figure 4C). The tested new sample is from the company with a same batch number, therefore forced us to think there is something wrong with the old sample, probably involved contamination. ABCFigure 4 Old human LF sample showed a cell death promoting effect on BL2 cells in nutrient deprived condition, but not reproduciable with a new sample. BL2 cells were taken from 50% x-vivo media and were grown in RSF supplemented with BSA. Apoptosis levels were assessed by flow cytometry following staining with AxV/PI. (A) Cells were seeded in plates with three different seeding concentration, human LF(10ug/ml) were added and leave the cells grew for 24h (n= 3). (B) Cells were seeded at a concentration of 1x106/ml and different concentration or sources of LF were added, leave the cells grew for 24h (n= 2). (C) Testing the effects of LF with a new sample under the same experimental condition as (B) described (n= 2).

Lactoferrin showed a death promoting effect on BL2 cells and MUTU I cells under nutrient deprivation condtion but no effect with an exposure to STS or UVB

Due to the unexpected results, a new sample of hLF was retested using the same ways to induce apoptosis. Because lactoferrin at sites of inflammation can reach as high as 200ug/ml [32], the newly tested concentrations of hLF were adjust to 10, 50, 100ug/ml under the nutrient deprived condition. Old hLF sample was still used as a comparision. BL2 cells in RPMI serum free media for 24h showed a good response, about 40% cells were triggered to death (Figure 5Ai). 10ug/ml old hLF showed a cell death promoting effect while the same concentration of new sample does not, which is consist with the results of last experiment. However, the viability of cells (Blue bar) decreased signicantly in the presence of high concentrations (50, 100ug/ml) of new hLF compared with the untreated group though there is no difference bewteen those two concentrations. Additionally, the increased concentration of recLF and bLF (50ug/ml) did not show any effect. The same effects were seen regardless of the majority of cell were dead at 48h (Figure 5Aii). The new hLF sample was used in the next experiments instead of the old sample. A similar experiment was carried out with the induction of apoptosis by STS for 2h in the presence of hLF and recLF from 5ug/ml to 50ug/ml. The induction of apoptosis was very successful, with 85% cells undergoing apoptosis at the examined time (Figure 5B). By adding hLF or recLF with a broad range, no apoptosis delaying or promoting effects have been seen in this case. A similar experiment was performed with an exposure to UVB instead of STS, and around 60% cells were undergoing apoptosis with UVB exposure for 10mins followed by an incubation for 2h (Figure 5C). Still no

diffirence was seen between the UT group and LF groups. AiAiiBCFigure 5 Retesting effects of human LF and recombinant LF with an increased concentration on BL2 cells survival using nutrient deprivation, STS or UVB treatment. BL2 cells were taken from 50% x-vivo media and grew them in RSF supplemented with BSA, human or recombinant LF were added from the incubation with RSF. Apoptosis levels were assessed by flow cytometry following staining with AxV/PI. (A) Cells grew in RSF for 48h with or without LF and viability of cells was checked by flow cytometry at 24h and 48h (n= 3). (B) Cells were treated with 1μM STS and incubated with or without LF for 2h (n= 2). (C) Cells were treated under UVB (100mJ/cm2) for 10mins and incubated with or without LF for 2h (n= 2). With an interest on the death promoting effects of hLF showed in nutrient deprivation condition rather than STS or UVB treatment, another Burkitt's lymphoma derived Epstein–Barr virus (EBV) positive cell line MUTU I was tested using the same apoptosis stimuli. Due to time constraint, MUTU I cells were cultured in complete media and taken from it to set up experiment. Dot plots of MUTU I cells on 0h showed more than 80% cells were alive at the beginning (Figure 6Ai), after 24h incubation in RSF media, a proportion of cells were induced apoptosis which showing a high binding of Annexin V (Figure 6Aii). However, by adding hLF (100ug/ml), a proportion of MUTU I cells showed a weaker binding which formed the middle peak. By looking at this proportion (AxV medium) of cells, about 40% cells were in viable gate while about 60% cells were in the non-viable gate. Meanwhile, about 40% cells were PI positive which can confirm that this proportion of cells should involve into Annexin V positive gate (Figure 6Aii). Nearly 20% MUTU I cells were undergoing apoptosis when

exposed to nutrient deprived condition for 24h, which made no difference by adding hLF, recLF and bLF at various doses. However, when cells were incubated for 48h, the presence of hLF at various doses showed a death promoting effect while the recLF and bLF did not (Figure 6Aiii). 60% and 40% MUTU I cells were undergoing apoptosis when exposed to STS or UVB for 2h (Figure 6B and Figure 6C). By adding hLF or recLF, no significant difference was seen between control group and LF groups under these two stimuli (Figure 6B and Figure 6C). All the results gathered on MUTU I cells are consist with what we have found on BL2 cells. MUTU 1 0hAiAiiAxV mediumAxV mediumNutrient deprivation 24hAiiiNutrient deprivation 48hBUV 2hCFigure 6 Testing effects of human LF and recombinant LF with an increased concentration on MUTU I cells survival using nutrient deprivation, STS or UVB treatment. MUTU I cells were taken from complete media and grew them in RSF supplemented with BSA, human or recombinant LF were added from the incubation with RSF. Apoptosis levels were assessed by flow cytometry following staining with AxV/PI. (A) Cells grew in RSF for 48h with or without LF and viability of cells was checked by flow cytometry at 24h and 48h (n= 2). (i) Light scatter profile and cell state of MUTU I. (ii) Annexin V histogram of total cell population, dot plots and PI histogram of AxV medium population of MUTU I cells in the presence of 100ug/ml hLF for 24h. (iii) The viability of cells in nutrient deprived condition incubated with or without LF (hLF, recLF, bLF) for 24h and 48h. (B) Cells were treated with 1μM STS and incubated with or without LF for 2h (n= 2). (C) Cells were treated under UVB (100mJ/cm2) for 10mins and incubated with or without LF for 2h (n= 1).

Discussion

Apoptosis is a programmed cell death, which plays a crucial role in tissue homeostasis by regulating the fate of cells. Unwanted cells undergo apoptosis are rapidly engulfed by neighbouring cells or professional phagocytes such as macrophages [33]. Due to the immediate clearance of the apoptotic cells or cell corpses, apoptosis often appears to be non-inflammatory or anti-inflammatory, which differs from the outcome of necrotic cells clearance. Many apoptotic cell associated molecules and macrophage or other cells associated mediators are involved in the regulation of this non-inflammatory clearance process, but the mechanism behind is not fully understood [34, 35]. Failure of cells to undergo apoptosis can lead to various diseases including cancer. In turn, more studies suggest that the apoptotic cell associated molecules played an important role for tumour growth in the tumour microenvironment (As reviewed in these papers[13][14]). Lactoferrin released by apoptotic lymphoma cells have been found as a keep out signal of neutrophils by inhibiting the migration ability [17]. Taking the evidence that neutrophils is often absent in tumour microenvironment, the migration inhibiting ability of LF might reveals a pro-tumour effect. First discovered in human milk half a century ago, lactoferrin has been well studied as an iron-binding glycoprotein which is associated with anti-microbial, anti-inflammatory and immunoregulatory properties [25, 26, 36, 37]. But the effects of LF on cell growth are contradictory, reported as both growth promoting and growth suppressing [19, 38, 39, 40]. In terms of tumour treatment, a mouse model showed a tumour suppressing effect by orally taken recombinant human LF [41]. A recombinant human lactoferrin

also showed an anti-tumour ability in phase I clinical trail [42]. Inconsist report of LF on tumour cells reveals a discreet choice of LF in cancer treatment, especially be critical to different type of cancer. The concentration of LF in blood is low, but it can reach as high as 200ug/ml at sites of inflammation [43]. However, no study has been reported on the physiological concentration of LF in tumour tissue in situ so far. By taking the evidence that apoptosis is frequent in BL cells and LF is released by apoptotic BL cells in situ, it could be reason that the concentration of LF in BL cells is between 0. 1ug/ml to 200ug/ml. According to it, physiological concentration of human milk derived lactoferrin, recombinant lactoferrin and bovine lactoferrin have been tested in this study. X-vivo 20 is a serum free media and it consisits of pharmaceutical grade human albumin, recombinant human insulin and pasteurized human transferrin. It was used to support cells grow in a serum free condition, which can deplete the effects of serum bovine LF. Trypan blue stains dead cells as blue but cannot distinguish live cells between apoptotic cells that is committed to die. In complete media, cells seeded at 4x105/ml entered into plateau phase on day 4 followed by a decrease in trypan blue negative cells. In contrast, in 50% x-vivo media, cells seeded at 4x105/ml seen an increase in total cell numbers while remain stable of trypan blue negative cells after day 4. At a lower seeding density, BL2 cells tend to have a longer lag phase in either complete media or 50% x-vivo media. Autophagy is another type of programmed cell death often induced in nutrient deprived condition through inhibition of mammallian target of rapamycin[44]. Cells maintain cellular energy level by break down their own cellular component during nutrient starvation through autophagy

[45, 46]. It suggusts the survival role autophagy played in nutrient starvation. In the nutrient deprivation experiment, only about 10% BL2 cells were undergoing apoptosis at 24h. DAPI staining was carried out to confirm cells died through apoptosis. Although DAPI staining cannot show cells undergo autophagy, it still confirms that at least the majority of cells were died through apoptosis from the stained bright blue framented DNA. Initialy, LF was expected as a survival factor for BL derived cells by delaying or inhibiting apoptosis. No effects have been shown with low concentration of human LF on different stimuli induced apoptosis experiments, not until 10ug/ml of hLF have been shown a death promoting effect on BL2 cells under nutrient deprivation condition. It was futher confirmed by a dose variance experiment. However, a new preparation of hLF from the same company of a same batch number does not show any death promoting effect under the same experimental condition. It could be reason that the old sample might have been contaminated, but no bacterial have been observed under the microscope when cells were incubated with the old sample of LF. The possible contamination can be tested with a bacterial growth experiment. In addition, the purity of hLF is only 85%, therefore the death promoting effect might comes from the foreign substances within the sample. A new sample of human LF from a different company with a higher purity could be used to figure out this suspicion in further work. However, in this study, the new hLF sample from the same company have been tested and showed a death promoting effect under the condition of nutrient deprivation, but not showed any effect with STS or UV treatment on BL2 or MUTU I. But the effect restricted with a high concentration (above 50ug/ml),

which is less relevant to physiological condition of the tumour microenvironment we interested. MUTU I is a BL derived Epstein–Barr virus (EBV) positive cell line. When exposed to STS or UVB, only a small proportion of MUTU I cells were induced apoptosis compared to BL2 cells with the same stimuli. It might because the MUTU I cells taken from complete media rather than the serum free media were more tolerant at the beginning with an exposure to an apoptotic inducer. Our group found that lactoferrin can bind to phosphatidylserine (data not published), which would be an explanation of the weaker binding of Annexin V in the presence of LF on MUTU I cells. PS is an inner membrane component which exposed to the surface of membrane when cells undergoing apoptosis [47]. Annexin V binds to PS with a high affinity to detect apoptotic cells, but the binding might be partially blockade by lactoferrin. However, the weaker binding of AxV is only seen on MUTU I cells, which suggusts the hypothesis need more investigation. In this study, recombinant lactoferrin and bovine lactoferrin does not show any effect under different stimuli on different cell lines. The comparison of human lactoferrin and recombinant lactoferrin in terms of two important abilities, iron binding and antibacterial activity, were investigated in mice model [48]. It showed the two lactoferrin are structurally and functionally similar. The different behave of hLF and recLF in this study might suggests the death promoting effect of hLF is independent of iron binding ability. The amino acid sequence of bovine lactoferrin only shares 68% similarity with human LF [49]. This difference offered many possibilities on the different behave between hLF and bLF on BL2 and MUTU I cells, which is currently unknown. In conclusion, lactoferrin does not have a survival effect on BL2 or MUTU 1

cells. But restricted by the cell types and stimuli in this study, some further work need to be done before making a final conclusion. Meanwhile, the death promoting effect showed with a high concentration need more investigation. Different Burkitt’s lymphoma derived cell lines and more apoptosis inducer with a different mechanism can be tried to figure out this question. In addition, cells can be taken from lag phase or plateau phase to set up each experment.

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