Knockdown of CD-74 in the Proliferative and Apoptotic Activity of Breast Cancer Cells

Hussain Al Saadh1, Waleed Al Abdulmonem2*, Zafar Rasheed3, Inamul Hasan Madar4, Jamila Alhodori1, Samah K. Nasr Eldeen1, Ali Alradhwan5, Noura Alasmael6, Abdullah Alkhamiiss7, Nelson Fernández1

1School of Biological Sciences, University of Essex, Colchester, UK; 2Department of Pathology, College of Medicine, Qassim University, Qassim, Saudi Arabia; 3Department of Medical Biochemistry, College of Medicine, Qassim University, Saudi Arabia; 4Department of Biotechnology and Genetic Engineering, Bharathidasan University, Tiruchirappalli, India; 5Clinical Laboratory Sciences, Inaya Medical College, Riyadh, Saudi Arabia; 6Central Laboratories, Egyptian Ministry of Health, Tanta, Egypt; 7Biochemistry Department, College of Medicine, Imam Abdulrahman Bin Faisal University, Saudi Arabia; 8College of Medicine, King Saud University, Saudi Arabia

Abstract

BACKGROUND: The cluster of differentiation (CD) 74 is known for its immunological functions and its elevated level was reported in various cancer cells.

AIM: The aim of the present study was to investigate the expression and potential roles of CD74 in the proliferative and apoptotic activity of breast cancer.

METHODS: Expression of CD74, macrophage migration inhibitory factor (MIF) and CD44 was assayed in CAMA-1 and MDA-MB-231 cell lines using flow cytometry. CD74 was knocked down using CD74 siRNA transfection in CAMA-1 and MDA-MB-231 cells and proliferation and apoptosis were determined in the transfected breast cancer cells.

RESULTS: The data showed that CD74, MIF and CD44 were expressed in breast cancer cell lines and were associated with cell proliferation and apoptosis. Correlation analysis revealed that CD74 was positively correlated and colocalised with MIF on the cell-surface of CAMA-1 and MDA-MB-231. The knockdown of CD74 significantly reduced CAMA-1 and MDA-MB-231 cell proliferation and increased the level of apoptotic cells.

CONCLUSION: We concluded that the interactions of CD74 with MIF and CD74 with CD44 could be a potential tumour marker for breast cancer cells. Moreover, the level of co-expression of MIF and CD74 or CD44 could be a surrogate marker for the efficacy of anti-angiogenic drugs, particularly in breast cancer tumours. In short, the study revealed the potential roles of CD74 in the proliferation and apoptosis of breast cancer which may serve as a potential therapeutic target for breast cancer.

Introduction

The role of a cluster of differentiation (CD) – 74 is a transmembrane glycoprotein, and its role has recently been reported in the pathogenesis of several cancers including breast cancer [1], [2]. Several studies have suggested that a small proportion of intracellular CD74 is modified by the addition of chondroitin sulfate (CD74-CS), a form of CD74 and chondroitin sulfate is a sulfated glycosaminoglycan usually found attached to proteins as part of a proteoglycan [3], [4]. CD74-CS is expressed on the surface of immune cells and can bind MIF, mediating MIF’s signalling pathway [3], [4]. Cell-surface expression of CD74 is not strictly dependent on the expression of class II MHC molecules in term of antigen presentation [5], [6] and numerous non-class II positive cells express CD74 which functions as a receptor for the initiation of different signalling cascades [7], [8]. MIF is the natural ligand of CD74 and binds to the extracellular domain of CD74 with high affinity (KD = 1.40 Å ~ 10⁻⁹ M) and initiates a signalling cascade [9]. When bound to the extracellular domain of CD74, MIF promotes signalling pathways including cell proliferation and apoptosis [9], [10], [11], [12], [13]. The short cytoplasmic tail of CD74 lacks an intracellular signal-transducing domain, although serine phosphorylation takes place in the P35 variant of CD74, requiring CD44, a
polymorphic transmembrane protein with kinase activating properties [14], [15]. CD74 forms a complex with CD44 which is essential for the MIF-induced signalling cascade [10], [16]. This cascade induces phosphorylation of ERK1 and ERK2 and activates various effector proteins involved in inflammatory processes and cell proliferation. ERK1 and ERK2 remain phosphorylated for many hours and hence this cascade continues for up to 2 to 3 hours [17], [18], [19], [20], [21]. Concurrently, MIF binding to CD74 activates the P13K-Akt pathway leading to phosphorylation of BAD and BAX proteins which are involved in apoptosis [22]. In addition, this cascade augments Bcl-2 expression, further supporting cell survival [23], [24], [25]. Thus, the binding of MIF to the CD74 / CD44 complex initiates a pathway resulting in the proliferation of the mature B cell population and their rescue from cell death. In addition to activating the P13K-Akt pathway, MIF binding to CD74 also induces a signalling pathway which involves Syk tyrosine kinase [6], [16] and induces cleavage of intramembrane CD74 regional releases intracellular domain (CD74-ICD) [26], [27]. CD74-ICD translocates to the nucleus where it induces activation of transcription mediated by the NF-κB p65 / RelA homodimer and its co-activator, TAFII105, resulting in regulation of transcription of genes that control B cell proliferation and survival [3], [6], [16]. Therefore, the CD74-MIF-CD44 complex initiates a pro-survival signal leading to the increase of proliferation and inhibition of apoptosis.

Recently, we quantified colocalization of CD74 and CD44 in breast cancer cells through non-invasive and validated biotyping procedure [28] and also determined several novel biomarkers involved in the pathogenesis of breast cancer [29]. Not only have these, but we also showed that treatment of human breast cancer cells with interferon-γ up-regulates the expression of CD74 along with MIF and CD44 [2], [30]. In continuation of these studies, the present study was hypothesised to find out the potential roles of CD74 in the proliferative and apoptotic activity in breast cancer. This was achieved by studying the colocalization of CD74 and MIF as well as CD74 and CD44 by two different techniques confocal microscopy and immunoprecipitation. The cells proliferation and apoptosis in CD74 siRNA transfected cells were also studied to address the hypothesis that blocking CD74 or MIF would affect apoptosis and cell proliferation.

Methods

Cell lines and cell culture

Two human mammary gland cell lines, CAMA-1 and MDA-MB-231, were used, which were derived from a malignant pleural effusion. The CAMA-1 cell lines were maintained in RPMI 1640 medium (LONZA-Belgium), supplemented with 10% (v/v) fetal calf serum (FCS; Imperial Laboratories, Andover, UK). The MDA-MB-231 cell line was maintained in D-MEM (high glucose), supplemented with 10% FCS. Raji cells (human negroid Burkitt’s lymphoma) and HeLa cells (human cervical cancer), expressing high levels of CD74, MIF, and CD44, respectively, served as additional positive controls. Raji and HeLa cells were cultured in RPMI 1640 (LONZA-Belgium) containing 10% FCS and cultured in a humidified atmosphere of 5% CO₂ at 37°C. All media used for this study were purchased from PAA Laboratories GmbH (Pasching, Austria).

Flow cytometry analysis

Cell lines were lifted with Accutase (Sigma Aldrich), and 1 x 10⁶ cells were used per sample. Monoclonal primary antibodies, By2 (anti-CD74), ab55445 (anti-MIF) and 156-3c11 (anti-CD44) were employed in indirect immunofluorescence staining. Cells were preincubated with saturating concentrations of primary antibody, followed by washing and labelling with FITC-conjugated goat anti-mouse IgG (Bio-legend). For cell surface staining, cells were fixed with 4% formaldehyde solution and washed with 1X phosphate-buffered saline (PBS). The cells were then blocked with blocking buffer (PBS / 0.1% BSA, bovine serum albumin) and washed in PBS. Primary and secondary antibodies were diluted with 0.1% BSA in PBS. Cells were sorted on a BD FAS Aria and analysed by FlowJo 8.8.6.

Immunofluorescence

In preparation for confocal immunofluorescence microscopy for studying colocalization between CD74 and MIF, CAMA-1 and MDA-MB-231 cells were cultured in LabTek 8-well chambers (Thermo Fisher Scientific) at a density of 6 x 10⁵ cells per well for two days. Following this, the cells were seeded. The cells were fixed with 4% paraformaldehyde for 20 min on ice. Cells were then blocked with 2% (w/v) BSA prepared in 1X PBS for 1 h at room temperature. For single staining of each antigen, cells were incubated with anti-CD74 (clone: By2) at a concentration of 1:500, (anti-MIF) (clone: ab55445) and 156-3c11 (anti-CD44) at a concentration of 1:400 for 1 h, followed by three washes with PBS. Secondary antibody, anti-mouse IgG conjugated with Alexa Fluor® 488 or Alexa Fluor® 555 (Invitrogen, Carlsbad, CA, USA), was used at a dilution of 0.25 µg / 100 ml for 1 h. For double staining, cells were blocked again with 2% BSA and the staining process was repeated for each desired pair. Cells were thoroughly washed with PBS, the chambers removed, and the slide was mounted with anti-fade mounting medium (Vector Shield) covered with a coverslip (Chance proper LTD, West Midlands, England) and sealed with rubber cement (Fixogum).
Rubber Cement, Marabu, Germany). Cells were incubated with a primary antibody followed by a secondary antibody. CD74 was labelled with FITC Alexa Fluor 488 (green) and CD44 was labelled with Alexa Flour 555 (red). Colocalization of CD74 with CD44 was assessed by Pearson’s correlation coefficient was used to analyze the degree of colocalization. The scale vary between -1 and 1, where 1 stand for colocalization, 0 stands for no colocalization and 0 stands for negative colocalization. 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) counterstain (Vector Laboratories, Burlingame, CA, USA) was used at a 1:250 dilution.

**Quantitative colocalization analysis of confocal fluorescence microscopy images**

To investigate whether CD74 and MIF colocalize, we used a high precision single-cell bioimaging protocol we employed, previously developed by our research group [31]. The Pearson correlation coefficient (PCC) was used for quantitative analysis of colocalization [31], [32]. PCC provides the overall association of two probes in an image, statistically. It also indirectly measures the quantity, i.e. the fraction of one protein that colocalises with another protein. A Nikon A1Si confocal microscope (Nikon Instruments Inc.) with a plan-apochromatic VC1.4 N.A. 60X magnifying oil-immersion objective was used for image acquisition. Images were acquired in three channels, using one-way sequential line scans. DAPI was excited at 398.7 nm with laser power 1.6 arbitrary units, and its emission was collected at 450 nm with a PMT gain of 86. Alexa Fluor 488 was excited at 488 nm with laser power 5.8; its emission was collected at 525 nm with a PMT gain of 117. Alexa Fluor 555 was excited at 560.5 nm with laser power 3.7, and its emission was collected at 595 nm with a PMT gain of 98. The scan speed was ¼ frames/s (Galvano scanner). The pinhole size was 35.76 μm, approximating 1.2 times the Airy disk size of the 1.4-NA objective at 525 nm. Scanner zoom was centred on the optical axis and set to a lateral magnification of 60 nm/pixel. Axial step size was 105 nm, with 80-100 image planes per z-stack.

**Small interfering (si) RNA transfection**

CAMA-1 and MDA-MB-231 cell lines were seeded in six-well plates at a density of 2 × 10^4 per well in 2 ml normal growth medium supplemented with 10% FCS. The cells were then allowed to grow until they reached 60-80% confluency. For each transfection, 4 µl of CD74 siRNA duplex at dose of 80 pmols (sc-35023) (Santa Cruz Biotechnology, USA) was diluted and 4 µl of siRNA transfection reagent was added at dose of 80 pmols (sc-29528) (Santa Cruz Biotechnology, USA) into 100 µl of siRNA transfection medium (sc-36868) (Santa Cruz Biotechnology, USA) separately without serum or antibiotics. Both diluents were mixed and incubated for 15-45 minutes at room temperature. Cells were then washed with 2 ml of siRNA transfection medium. The siRNA transfection reagent mixture was then added to each well, and the volume made up to 1 ml by adding 800 µl of siRNA transfection medium (Santa Cruz Biotechnology, USA). In the same manner, this was applied for negative control siRNA (sc-44230) (Santa Cruz Biotechnology, USA). The cells were then incubated overnight at 37°C in a CO2 incubator for 18-24 hr. Following incubation, the medium was aspirated and replaced with fresh 1X normal growth medium. Cells were assayed using the appropriate manufacturer’s protocol 24-72 hours after the addition of fresh medium in the step above. Transfection efficiency was confirmed by western blot and microscopy. Once the transfection was confirmed, the effect of CD74 siRNA on the proliferation and apoptosis of CAMA-1 and MDA-MB-231 could then be studied.

**Proliferation and Apoptosis assay**

CAMA-1 and MDA-MB231 cell lines were cultured in 6 well plates at a density of (15 × 10^4 cell/well) or in 96 well plates at a density of (15 × 10^2 cell/well) at 37°C and then transfected with CD74 siRNA duplex as explained previously. The cells were then washed twice with 1X PBS and incubated with 2 µl of Annexin V-FITC (BioLegend, UK) at room temperature for 20 minutes in the dark. Cells were then thoroughly washed and then fixed with 4% PFA followed by washing steps in PBS. Finally, the samples were read using BD FACSARia and analysed by FlowJo 8.8.6. In the same manner, the MTT assay was then used to assess cell proliferation. Briefly, 20 µl of MTT solution (5 mg/ml in PBS) and 100 µl was added per well, and cells were incubated at 37°C with 5% CO2 in a humidified chamber for 4 hr for colour development. The resultant Formazan crystals were dissolved in dimethyl sulfoxide (100 µl) and the absorbance intensity measured at 595 nm using a microplate reader (Versamax). The percentage of cell proliferation was calculated relative to the rate of proliferation in untreated cells.

**Results**

**Cell-surface expression of CD74, MIF and CD44**

The cell-surface expression of CD74, MIF and CD44 were analysed in CAMA-1, MDA-MB-231. Non-permeabilized were stained with an appropriate concentration of By2 (anti-CD74), ab55445 (anti-MIF) and 156-3C11 (anti-CD44) antibodies followed by 1 µl RAM-FITC secondary antibody. Cells without staining and isotype cells, stained with only secondary
antibody, were used as a negative control. CD74, MIF and CD44 expression were detected on the cell surface and cytoplasmic of CAMA-1 and MDA-MB-231. Monocytes, Raji cells, cervical cancer HeLa cells, and lymphocytes, (Jurkat) cells, were used as a positive control as they express high levels of CD74, CD44, and MIF respectively. This is displayed in Figure 1 in where empty histograms show CD74, MIF or CD74 protein grey filled the histogram.

**Colocalization of MIF with CD74 and CD44**

To investigate whether MIF colocalised with CD74 or CD44 on CAMA-1 and MDA-MB-231 cells, all cell lines were immunostained with an appropriate primary antibody followed by a secondary antibody (Figure 2 and Figure 3).

**Knockdown of CD74 expression in CAMA-1 and MDA-MB-231 cells by CD74 siRNA**

Prior studies have reported that CD74 is overexpressed in human breast adenocarcinomas, and has a role in tumour progression along with MIF and CD44. The expression of CD74 in CAMA-1 and MDA-MB-231 cells was therefore evaluated. The expression of CD74 was found to be highest in CAMA-1 cells compared to MDA-MB-231 cells (Figure 4). In pilot experiments, it was found that a
concentration of 80 pmol/ml for 24 hr of specific CD74 siRNA was optimal for disrupted expression of CD74. Therefore, a dose of 80 pmol/ml was selected for optimal transfection of CAMA-1 and MDA-MB-231 cells with siRNA for all subsequent experiments.

Knockdown of functional CD74 expression in CAMA-1 and MDA-MB-231 cells promotes apoptosis

In the light of the observations indicating apoptotic modes of cell death in CAMA-1 and MDA-MB-231 cells treated with CD74 siRNA next, multiparameter flow cytometric analysis of siRNA-transfected CAMA-1 and MDA-MB-231 cells was pursued to obtain more sensitive and quantitative details of a possible apoptotic mode of cell death. Following 24 hr of a culture of CD74 siRNA-transfected CAMA-1 and MDA-MB-231 cells, flow cytometry was used to detect the expression of annexin V in the absence of PI staining.

Annexin V is a non-quantitative probe used to detect phosphatidylserine expressed on the cell surface, an indication of apoptosis. CAMA-1 and MDA-MB-231 cells treated with CD74 siRNA displayed significantly higher levels of annexin V staining (± 55% and ± 58% respectively) compared with negative control siRNA-treated counterparts (± 8% and ± 13% respectively) (Figure 5A). These observations indicate that CD74 might play important regulatory roles in apoptosis.

Effects of CD74 knockdown on CAMA-1 and MDA-MB-231 cell proliferation

CAMA-1 and MDA-MB-231 cell proliferation and viability were determined using the MTT metabolic and viability assay (Figure 5B). CAMA-1 and MDA-MB-231 cells treated with CD74 siRNA displayed significantly reduced proliferation compared to cells treated with the negative control siRNA control sequence.

Discussion

The present study aimed to investigate the role of CD74 and its interrelation to MIF in breast cancer cells. This was achieved by studying the expression and colocalization of MIF with CD74 and CD44 molecules in the breast cancer cell lines CAMA-1 and MDA-MB-231 cells. The results obtained from confocal microscopy demonstrated that CD74 and MIF are highly colocalized on the cell-surface of all...
breast cancer cells. Pearson’s correlation coefficient and scatter plot analysis (Figure 3) also gave rise to the colocalization of CD74 and MIF [33], which accurately depicts the percentage of colocalization of CD74 and MIF molecules. Several groups have studied the association of CD74 with MIF and CD44 in cancers since it was reported that CD74 and CD44 are involved in signalling with MIF [10, 15, 16]. We also showed that CD74 and CD44 colocalise in breast cancer cells using a non-invasive and validated bioimaging procedure [28]. Also, it was shown that the formation of a molecular complex between MIF, CD74 and CD44 in prostate carcinoma cells lines (DU-145) could mediate signal transduction including (gene regulation, apoptosis, and cell proliferation) in prostate cancer [34].

Previous studies by immunofluorescence have confirmed the colocalization of MIF and CD74 in non-small cell lung cancer [35]. Additionally, using correlation analysis, Zheng et al. identified a positive correlation between MIF and CD74 in gastric cancer cells [36]. Correspondingly, Starlets et al. showed that, in malignant B cells obtained from patients with chronic lymphocytic leukaemia (CLL), MIF binds to the extracellular domain of CD74 to initiate a signalling cascade leading to cell proliferation and survival [6]. The interaction of MIF with CD74 and CD44 has been reported, suggesting that MIF in association with CD74 and CD44, as a complex, plays a significant role in bladder cancer cell proliferation [37]. Similarly, Meyer-Siegler et al., reported that the interaction between MIF and CD74 activates the ERK1 and ERK2 signalling pathway, presumably through interaction with CD44, in the prostate cancer cell lines DU-145 and LNCaP, but not in normal human prostate epithelial cells (PrEC) or benign prostate epithelial cells (BPH-1) [34]. However, human benign prostate hyperplasia epithelial cells (BPH-1) and PrEC prostate cancer cells do not express CD74 on the cell surface, so for this reason, both cells do not interact with MIF and CD44 [34]. Correspondingly, Shi et al. showed that mammalian COS-7 cells do not bind MIF unless engineered to express the extracellular domain of CD74 [10].

To investigate the role of CD74 in apoptosis and proliferation, siRNA that targeted CD74 was used. Western blot results (Figure 4A and 4B) showed that CD74 expression was strongly knocked down in CAMA-1 and MDA-MB-231 cells in comparison with the control and CD74 siRNA. Microscopic results also confirmed that CD74 expression was strongly knocked down in both cell lines (Figure 4C and 4D). When CD74 expression was knocked down, apoptosis was observed in CAMA-1 and MDA-MB-231 cells. Both cell lines, when treated with CD74 siRNA, displayed significantly higher levels of annexin V staining (± 55% and ± 58% respectively) compared to negative control siRNA-treated counterparts (± 8% and ± 13% respectively). In the same manner, it was found that in CAMA-1 and MDA-MB-231 cells treated with CD74 siRNA, a significantly reduced proliferation was observed compared to cells treated with the negative control siRNA control sequence and untreated cells. Likewise, it has been reported that knockdown of MIF or CD74 expression by RNA interference inhibits DU-145 cell proliferation and downstream MIF signalling [34], [38]. It is also reported that knockdown of the functional expression of MIF markedly decreased H460 cell proliferation and induced apoptosis, as seen by augmented expression of annexin A5 following treatment of H460 cells by MIF siRNA [39]. In particular, Verjans et al. showed that anti-MIF and anti-CD74 antibodies potently blocked cell proliferation of non-invasive MDA-MB-468 and invasive MDA-MB-231 breast cancer cells; however, this was not observed in non-tumorous MCF-12A cells [40]. This could be explained by the absence of the cell-surface portion of CD74 in MCF-12A cells. It is also reported that CD74 regulates Fas death receptor signaling in lymphomas by decreasing the levels of Fas receptors on the cell surface [41]. In the same manner, Liu et al., have shown that CD74 promotes tumor growth, angiogenesis, and cancer cell metastasis in vivo [42]. The effect of CD74 in tumor growth and cell proliferation was studied by blocking the activity of MIF or CD74 in HEK / CD74 or a renal cell carcinoma (Caki-1) cells. The data showed that CD74-upregulated vascular endothelial growth factor D (VEGF-D) positively regulates the expression of cyclin D and E, which results in the promotion of cell cycle progression [42]. It was reported that G1 / S phase proteins cyclin D and cyclin E were upregulated by CD74 and promoted cell cycle progression [6]. The recent finding also showed that the expression of CD74 was associated with MIBC / high grade of the UCB, while the knockdown of CD74 attenuated the proliferation, invasion, and angiogenesis of HT-1376 [18]. Figure 6 shows the proposed signal transduction pathway of MIF with CD74 and CD44.

In conclusion, it was observed that the interaction of MIF with CD74 CD44 could be a potential tumor marker for breast cancer cells. Moreover, level of co-expression of MIF and CD74 could be a surrogate marker for the efficacy of anti-angiogenic drugs, particularly in breast cancer tumors. Also, knockdown of CD74 by CD74 siRNA significantly reduced CAMA-1 and MDA-MB-231 cell proliferation and increased the level of apoptotic cells.

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