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MICA Regulates the Expression of DAP10 and Signals through an Independent PI3K Pathway in NKG2D Positive Cervical Cancer Cells

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Abstract

NKG2D receptor engages ligands such as MICA and MICB, which activates cytotoxicity in NK cells leading to the destruction of tumour cells expressing these ligands. In normal human lymphoid cells the association of DAP10 with NKG2D is essential for signalling and important for its cell surface expression. However, the mechanism of the NKG2D/DAP10 complex upregulation is not completely understood in cancer. Also, the role of DAP10 in the activation of the PI3/AKT signaling pathway in cervical cancer has not been fully elucidated. In the present study, we investigated the role of MICA in the regulation of DAP10 in cervical cancer cells. First, we demonstrate the presence of the NKG2D/DAP10 complex in different tumour cell lines by flow cytometry. Also, we demonstrate that MICA upregulates the expression of DAP10 in cervical cancer cells in a time dependent manner by immunoblotting. We found that the AKT kinase is constitutively phosphorylated and MICA induced an increase in tyrosine phosphorylation. Furthermore, this activation is independent of the PI3K in cervical cancer cell lines as determined by immunoblotting and flow cytometry. Our results provide evidence supporting the notion that MICA functions as a stimulatory molecule to regulate the expression of the receptor adapter DAP10 in cervical cancer cells and thus may contribute to their proliferation and survival. The possibility that the NKG2D-DAP10 complex is widely expressed in different types of cancer may confer an advantage to transformed cells to survive in the tumour microenvironment and escape from the immune surveillance.

Keywords: MICA; NKG2D; DAP10; PI3K; AKT; Cervical cancer

Introduction

NKG2D is an activating receptor mainly present in NK and different subsets of lymphoid cells that engage ligands such as the stress-induced molecules HLA class-I chain-related A (MICA) and MICB (1) [1,2], which activates cytotoxicity in NK cells through their ligation to NKG2D [3,4], leading to the destruction of tumour cells expressing MICA and MICB ligands [5-7]. It has been shown in normal lymphoid cells that the association of adapter molecules, such as DAP10 in humans, with NKG2D is essential for signalling and considered important for its cell surface expression [8,9].

DAP10 is a transmembrane protein with conserved aspartic acid residues in their transmembrane regions that interact with a positively charged arginine residue in the transmembrane region of NKG2D. The pairing of NKG2D with DAP10 adapter proteins is a unique feature of murine NKG2D, as the human NKG2D receptor has been shown to be structurally incapable of associating with DAP12 [10]. Signal transduction through DAP10 is achieved by the intracellular YxxM motif that, upon tyrosine phosphorylation recruits PI3K or the growth factor receptor-bound protein 2/VAV1 signaling intermediate, thereby activating protein kinase AKT, as well as ERK and JNK in MAPK cascades [11]. These branched signalling pathways ultimately trigger lymphocyte cytotoxic granule polarization and degranulation, cytokine production, proliferation, and survival. Stable surface expression of NKG2D is dependent on its association with DAP10, which is mediated by oppositely charged amino acid residues in their transmembrane domains. The apparent requirement for co-expression of an adapter protein for NKG2D cell surface expression is further highlighted by a study showing that COS-7 cells transiently transfected with porcine NKG2D require DAP10 co-transfection to achieve cell surface expression of NKG2D [12].

Cell surface expression of NKG2D and DAP10 can be both positively and negatively regulated by cytokines. Several independent studies have shown that human and murine $CD8^+$ T cells stimulated with IL-2 can rapidly increase both NKG2D and DAP10 expression [13-15]. IL-15 is a cytokine that drives proliferative and effector functions of both CTL and NK cells and recent observations have shown that it can induce NKG2D and DAP10 expression and help to activate NK cells to clear the cryptosporidium intestinal protozoa [16]. IL-15 has also been demonstrated to induce the cell surface expression of NKG2D on $CD8^+$ T cells, which expands the means by which these cells can be co-stimulated in the tissue microenvironment [17]. Upon NKG2D co-stimulation of TCR stimulated human naive $CD8^+$ T cells, the homeostatic cytokines IL-7 and IL-15 have been reported to maintain NKG2D and DAP10 expression [13]. In addition, IL-15 stimulation in conjunction with TNFα, has also been shown to induce an unusual subset of $NKG2D^+$ $CD4^+$ T cells which have been described in patients with rheumatoid arthritis [18].

Tumorigenic transformation of normal cells often leads to the induction of NKG2D ligands that serve as cellular stress signals [19]. The presence of soluble MICA/B appears to be a common occurrence for tumors expressing these NKG2D ligands [20-22]. So, although initial expression of MICA/B may result in the removal of tumor cells,

the shedding of MICA/B may be a trait selected within the tumor cell population to avoid immune recognition. However, the regulating mechanisms of DAP10 expression in tumour cells are not clear.

Previously we have demonstrated that epithelial cervical cancer cells express NKG2D receptor, MICA/MICB and respond to MICA/MICB to enhance their proliferative behaviour [23]. Therefore, we analysed the regulation of DAP10 by MICA in NKG2D positive cervical cancer cells. Here, we present that positive NKG2D cervical cancer cells also express DAP10 and this expression is upregulated by the same ligands. Furthermore, we demonstrate that upon ligation NKG2D signals through AKT, which is independent of the PI3K pathway in cervical cancer cells.

Materials and Methods

Cells and antibodies

The HeLa, Hep2G and MDA231 cell lines were purchased from ATTC whereas INBL and CALO cell lines were established in our laboratory [24]. Tumour cell lines preserved in our laboratory were cultured at 37°C with 5% CO_2 in RPMI-1640 (Gibco) supplemented with 10% heat-inactivated Foetal Calf Serum (FCS) (Gibco), 100-U/ml penicillin and 100-g/ml streptomycin (Gibco). Polyclonal antibody against MICA/MICB and murine monoclonal anti-MICA, anti-MICB, anti-NKG2D, anti-pAKT (Ser473) and anti-AKT antibodies were purchased from Santa Cruz Biotech (California, USA); human recombinant MICA and MICB from R&D Systems (R&D Systems MN, USA). Secondary antibodies were purchased from Zymed Laboratories. (San Francisco California, USA).

Stimulation with MICA or MICB

CALO cells were seeded in Petri dishes (2×10^6 cells per dish) (Thermo Fisher, Roskilde, Denmark) and incubated with RPMI 1640 containing 0.5% FCS (Invitrogen, Carlsbad, CA, USA) for 24 hours at 37°C to deprive cells of growth factors. Cells were then incubated with RPMI 1640 without FCS for 15 min and subsequently stimulated with 10 IU/mL or 100 IU/mL of human recombinant MICA (R&D Systems, Minneapolis, MN, USA) for different periods of time.

Cell lysis

Cells were lysed with ice-cold lysis buffer [1% Triton X-100, 5 mM EDTA, 140 mM NaCl, 50 mM Tris (pH 7.4), 1 mM PMSF, 1 mM NaF, 1% aprotinin, 1μM leupeptin, 1 μM pepstatin and 100 μM Na_3VO_4] for 15min. Lysates were clarified by centrifugation at 13,000 rpm at 4°C for 15 min, and the supernatants were then collected.

Immunoprecipitation and immunoblotting

For immunoprecipitation, treated or untreated cells were lysed as mentioned above. The total protein content of the lysates was determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA), and 150μg of protein were incubated with protein A-agarose beads (Invitrogen, Carlsbad, CA, USA) previously coupled with anti-DAP10 or anti-pAKT antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 3 hours at 4°C. Immunoprecipitated proteins were washed five times with ice-cold lysis buffer, resolved by 15% SDS-PAGE and transferred to nitrocellulose membranes (Trans-Blot, Bio-Rad, CA, USA). Membranes were blocked in Tris buffered-saline with 0.1% Tween 20 (TBST) and 2% bovine serum albumin overnight at 4°C.

Membranes were analysed using mouse anti-pAKT antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation with a horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibody (Zymed Laboratories, San Francisco CA, USA). Proteins were visualized by using the enhanced chemiluminescence detection system (Super Signal, Pierce, Rockford IL, USA). After stripping with 0.1M glycine (pH 2.5) and blocking, the same membranes were incubated with the anti-DAP10, anti-PI3K or anti-AKT antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and HRP-conjugated secondary antibody. All figures show representative results of at least three independent experiments.

Flow cytometry

Cell suspensions (0.4×10^6 cells/ml) in PBS with 5% FCS and 0.01% azide were incubated with 10 μg/ml of the primary murine monoclonal anti-NKG2D, anti-MICA or anti-DAP10 antibodies or the respective isotype control for 90 min at 4°C. After washing the cells with PBS, they were incubated in the dark for 30 min with 0.45-μg/ml of FITC-labeled goat anti-mouse IgG at 4°C. After washing again, the cells were fixed for 20 min in 1% paraformaldehyde, followed by two more washes. The stained cells were analysed in a FACS Aria II cytometer (Becton Dickinson).

Results

Expression of MICA, MICB, NKG2D and DAP10 on cancer cells

The expression of MICA and MICB by tumour cells has been previously reported [5]. Additionally, our previous results provide evidence for the ability of cervical cancer cells to express NKG2D receptor [23].

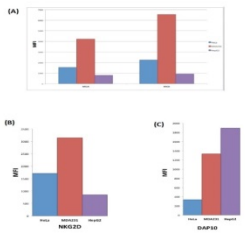

Figure 1: Presence of MICA, MICB, NKG2D and DAP10 in different tumour cell lines. 0.4×10^6 cells were incubated in the presence of the specific monoclonal antibodies for 90 min. (A) Antibodies anti-MICA or MICB; (B) Antibodies anti-NKG2D; (C) Antibodies anti-DAP10. After washing, the cells were incubated in the dark for 30 min with 0.45-μg/ml of FITC-labeled goat anti-mouse IgG at 4°C. Then, the cells were fixed for 20 min in 1% paraformaldehyde and washed with PBS. The stained cells were analyzed in a FACS Aria II cytometer.

A very important mechanism adopted by tumour cells to escape immune surveillance is the expression of the NKG2D receptor that has been implicated in tumor proliferation [23,25]. Hence tumour cells

HeLa, MDA231 and HepG2 were screened for the over-expression of NKG2D and associated molecules using flow cytometry. Whereas MDA231 cells over-expressed MICA and MICB, HeLa and HepG2 expressed low levels (Figure 1A). On the other hand, tumour cells MDA231 and HeLa were found to over-express NKG2D, while HepG2 cells express very low levels of NKG2D (Figure 1B). By flow cytometry analyses MDA231 and HepG2 cells were found to significantly express the adapter molecule DAP10 whereas HeLa expressed very low levels of DAP10 (Figure 1C). Therefore, the expression of the NKG2D system on tumour cells is a more general property and reflects the oncogenic pathways and immune escape strategies employed by malignant cells.

Expression of DAP10 in cervical cancer cells

After establishing that several tumour cells express the NKG2D receptor and its ligands we proceeded to determine the presence of DAP10 complex in cervical cancer cells by using specific antibodies. By immunoblot analyses C33, HeLa, CALO and INBL tumour cells were found to express DAP10 protein. C33, HeLa and INBL cells expressed low levels of DAP10 while CALO cells expressed high levels of the protein (Figure 2). A higher molecular weight protein was observed only in CALO cells. THP-1 cell line was used as a positive control since it has a myeloid origin and naturally expresses the NKG2D associated molecules.

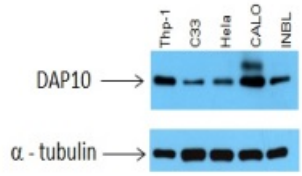

Figure 2: Expression of DAP10 in cervical cancer cells. 3 × 10^6 cervical cancer cells were lysed; DAP10 proteins were immunoprecipitated, separated on a 15% SDS-PAGE, transferred onto PVDF membranes and blocked with BSA overnight. Membranes were incubated in the presence of the specific monoclonal anti-DAP antibodies for 90 min and for 30 min with the secondary antibody. DAP10 was visualised by chemiluminescence. The myeloid THP-1 cell line was used as a positive control.

MICA regulates the expression of DAP10 in cervical cancer cells

After establishing that several cervical tumour cells express the NKG2D-DAP10 complex, we sought to investigate the effect of MICA stimulation on DAP10 expression in cervical cancer cell line CALO since the cells express a high amount of this protein. As shown in (Figure 3), DAP10 expression is down regulated at short times and increases to reach the maximum level at 24 hr.

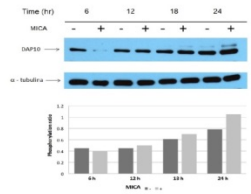

Figure 3: MICA regulates the expression of DAP10 in cervical cancer cells CALO. 2 × 10^6 cells per dish were incubated in the presence of MICA for different periods of time. After stimulation cells were lysed, DAP10 proteins were immunoprecipitated, separated on a 15% SDS-PAGE, transferred onto PVDF membranes and blocked with BSA overnight. Membranes were incubated in the presence of the specific monoclonal anti-DAP antibodies for 90 min and for 30 min with the secondary antibody. DAP10 was visualised by chemiluminescence.

PI3K/AKT signalling after NKG2D ligation in cervical cancer cells

It has been suggested elsewhere that the NKG2D-DAP10 complex on tumour cells could engage NKG2D ligands on adjacent tumour cells and lead to the activation of oncogenic PI3K-AKT signalling axis [25,26] therefore we analysed the activation of AKT by incubating cervical cancer cells with MICA or MICB.

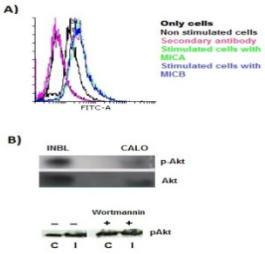

Figure 4: Activation of AKT after NKG2D ligation in cervical cancer cells. (A) 1 × 10^6 cells were incubated in the presence of MICA or MICB overnight. Cells were harvested, permeabilized with methanol and incubated in the presence of anti-pAKT antibodies (Ser473) or anti-AKT antibodies, washed and incubated in the dark for 30 min with FITC-labeled goat anti-mouse at 4°C. Then, the cells were fixed for 20 min and washed with PBS. The stained cells were analysed in a FACS Aria II cytometer. (B) 5 × 10^6 cells (C=CALO) (I=INBL) were incubated in the presence of wortmannin (100 nM) for 1 hour. Cells were lysed and phosphoAKT was immunoprecipitated, separated on a 10% SDS-PAGE and electrotransferred onto nitrocellulose. Membranes were incubated in the presence of the anti-pAKT (Ser473), and proteins visualised by chemiluminescence. To visualise total AKT, membranes were stripped and blocked overnight with 3% BSA in TBST. Then, membranes were incubated with anti-AKT antibodies and AKT was visualised by chemiluminescence.

Our results show that AKT is constitutively activated in non-stimulated INBL and CALO cell lines and that tyrosine phosphorylation is increased in response to MICA and to MICB (Figure 4A). Also, we found that AKT is more abundant in INBL cells compared to CALO cells. To further analise the participation of PI3K in the activation of AKT we incubated CALO and INBL cells in the presence of wortmannin, an inhibitor of PI3K. We found that phosphorylation of AKT was not affected by the inhibitor suggesting that PI3K is not involved in its activation (Figure 4B).

Discussion and Conclusion

In normal cells MICA or MICB are absent and upon stress and malignant transformation its expression is induced. Most solid tumours and leukaemia constitutively express at least one NKG2D ligand and therefore are susceptible to immune-surveillance mediated by NKG2D. Although MICA and MICB are frequently co-expressed, some tumour cell lines carry only one of them on the cell surface. [27-31]. In the present investigation we have found that irrespective of their tissue origin, all cancer cell lines heterogeneously expressed NKG2D and the associated ligands. Interestingly, the cell lines preferentially express MICB. Also, we have found that all cancer cell lines showed a constitutive expression of DAP10. Particularly, cervical cancer cell lines expressed the NKG2D-DAP10 complex. It seems that the presence of both ligands and the receptor does not imply that the same amount of the adapter protein will be present in tumour cells. The differential expression of NKG2D and its ligands raises the question of whether they could regulate their own expression, or better yet, the expression of the receptor associated molecule DAP10. In normal T cells, NKG2D lacks intrinsic cytoplasmic signalling motifs. Therefore, it depends solely on the trans-membrane adapter DAP10 for cell-surface expression and function [26,32]. At this point, there is no hint of mechanisms of gene regulation that may underlie cancer cell expression of NKG2D and DAP10, which might be induced by cytokines or growth factors in transformed cells. In fact, our previous results point out that tumour cervical cancer cell lines can express NKG2D receptor but the question remained if DAP1O adaptor protein was also present in tumour epithelial cancer cells [23]. In this study we have shown that DAP10 is present in cervical cancer cells and interestingly that MICA up regulated the expression of DAP10 in a time dependent manner in these cancer cell lines. In human NK cells, the NKG2D-DAP10 complex is regulated by cytokines of the common chain (c) family (namely IL-2, IL7, IL-15 and IL-21) [32]. However, in non-lymphoid cells the mechanism of DAP10 regulation remains elusive. Our results provide evidence supporting the notion that MICA functions as a stimulatory molecule to regulate the expression of the receptor adapter DAP10 in cervical cancer cells and thus may contribute to their proliferation and survival. Further studies are required to explore the transcriptional control of the DAP10 expression. Understanding how the expression of NKG2D-DAP10 is regulated in tumour cells could have implications for immunotherapy.

It has been shown that the NKG2D-DAP10 complex on tumour cells could engage NKG2D ligands on adjacent tumour cells and lead to the activation of oncogenic PI3K-AKT signalling axis and downstream effectors [26]. Tumour cells could induce the expression of NKG2D to complement its ligands for self-stimulation that trigger oncogenic signalling cascades that promote tumour growth. Upon ligand binding, DAP10 is phosphorylated by a Src family kinase. Lck can sub-serve this function [33], but other Src-family members may also play this role [34]. The phosphorylated NKG2D-DAP10 complex can potentially bind multiple adaptor proteins, one of which is the p85a regulatory subunit of PI3K [9,35]. NKG2D stimulation leads to PI3K dependent AKT phosphorylation, which activates cell survival pathways [36,37]. We have found that in cervical cancer cells AKT is constitutively phosphorylated and the phosphorylation is increased in response to MICA or MICB when binding to NKG2D. Furthermore, that upon ligation NKG2D/DAP10 complex signals through an independent PI3K pathway since AKT activation was not abolished by wortmannin, which is a specific inhibitor of PI3K. It has been shown that PI3K activity is also required for optimal NKG2D-mediated calcium release and cytotoxicity [35,38,39], although the precise mechanism for this is unclear because PI3K activity is not required for DAP10 initiated tyrosine phosphorylation of Vav1 [33,35], PLC- [33,35] or SLP-76 [35]. Thus, it is possible that in cervical cancer cells other kinases may be recruited to phosphorylate AKT to maintain active the cell survival pathway. AKT isoforms contain PH domains with similar specificities for the D3-phosphorylated phosphoinositide products of PI3K [40]; they are regulated by phosphoinositide-dependent kinase-1 (PKD1), a PH domain-containing kinase downstream of PI3K, which phosphorylates AKT isoforms on a critical threonine residue in the activation loop [41]. Further investigation is required to identify different kinases that can activate AKT in cervical cancer cells.

The question arises as to whether NKG2D imparts cellular effects in cancer environments that are distinct from those observed in normal T cells. We observed stimulatory effects of recombinant MICA in tumour cell lines that might be activated through the adaptor molecule DAP10 to induce the activation of AKT through an independent PI3K pathway. Tumour cell lines may down-modulate NKG2D-DAP10 as an escape from detrimental effects of ligand-mediated self-stimulation under in vitro culture conditions. The possibility that the NKG2D-DAP10 complex is widely expressed in different types of cancer may confer an advantage to transformed cells to survive in the tumour microenvironment and escape from the immune surveillance.

Conflicts of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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