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Blocking oncogenic RAS enhances tumour cell surface MHC class I expression but does not alter susceptibility to cytotoxic lymphocytes

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ABSTRACT

Mutations in the RAS family of oncogenes are highly prevalent in human cancer and, amongst its manifold effects, oncogenic RAS impairs the expression of components of the antigen presentation pathway. This allows evasion of cytotoxic T lymphocytes (CTL). CTL and natural killer (NK) cells are reciprocally regulated by MHC class I molecules and any gain in CTL recognition obtained by therapeutic inactivation of oncogenic RAS may be offset by reduced NK cell activation. We have investigated the consequences of targeted inactivation of oncogenic RAS on the recognition by both CTL and NK cells. Inactivation of oncogenic RAS, either by genetic deletion or inactivation with an inducible intracellular domain antibody (iDAb), increased MHC class I expression in human colorectal cell lines. The common RAS mutations, at codons 12, 13 and 61, all inhibited antigen presentation. Although MHC class I modulates the activity of both CTL and NK cells, the enhanced MHC class I expression resulting from inactivation of mutant KRAS did not significantly affect the *in vitro* recognition of these cell lines by either class of cytotoxic lymphocyte. These results show that oncogenic RAS and its downstream signalling pathways modulate the antigen presentation pathway and that this inhibition is reversible. However, the magnitude of these effects was not sufficient to alter the *in vitro* recognition of tumour cell lines by either CTL or NK cells.

1. Introduction

The evasion of immunity is now recognised as a hallmark of cancer alongside well established characteristics, such as loss of growth control, resistance to apoptosis and the ability to invade and metastasise (Hanahan and Weinberg, 2011). Cancer development is initiated by mutations in cellular oncogenes and tumour suppressor genes. The first cellular oncogenes to be discovered were those belonging to the RAS family and mutant RAS molecules are found in 20–25% of human cancers (Karnoub and Weinberg, 2008; Pylayeva-Gupta et al., 2011). RAS proteins are small GTP-binding proteins that couple growth factor receptors to intracellular signalling pathways (Karnoub and Weinberg, 2008; Pylayeva-Gupta et al., 2011). Mutations in codons 12, 13 and 61 are the commonest in human cancer and all generate constitutively active RAS molecules (Pylayeva-Gupta et al., 2011). The RAS pathway is directly linked to several key features of malignant development, such as cell cycle progression and survival. In addition, RAS signalling regulates several features associated with invasiveness and the development of the tumour microenvironment, including tumour angiogenesis (Karnoub and Weinberg, 2008; Pylayeva-Gupta et al., 2011).

Cytotoxic lymphocytes, namely natural killer (NK) cells and cytotoxic T cells (CTL), play a key role in the elimination of tumour cells (Vesely et al., 2011). The activity of both of these cell types is regulated by target cell MHC class I molecules (Ljunggren and Karre, 1990; Lanier, 2008). The T cell receptor (TCR) complex on CTL can recognise tumour-associated antigens (TAA) presented by MHC class I molecules and this interaction delivers a potent activating signal to the cognate CTL. In contrast, NK cells are negatively regulated by MHC class I molecules *via* the interaction with inhibitory killer cell immunoglobulin-like receptors (KIRs). Thus,

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whilst reduction of MHC class I expression on tumours allows evasion of CTL, it favours NK cell activation, providing the host with a powerful immune surveillance system. In addition, malignant cells frequently express cell surface ligands of the NKG2D and DNAM-1 molecules found on both NK cells and CTL. In NK cells, these deliver signals that cause NK cell activation in the absence of inhibitory signalling from KIR molecules (Lanier, 2008; Vivier et al., 2011). Once activated by tumour cells, CTL and NK cells exocytose cytotoxic granules containing granzymes and perforin molecules, inducing apoptosis in the tumour. In addition, both NK cells and CTL produce interferon (IFN)-γ which enhances antigen presentation and favours the development of cell mediated immunity (Chowdhury and Lieberman, 2008; Krzewski and Coligan, 2012; Schoenborn and Wilson, 2007; Schroder et al., 2004).

Tumours frequently downregulate the expression of MHC class I molecules from the cell surface, allowing them to evade CTL recognition (Campoli and Ferrone, 2008). This can occur as a result of mutations in genes encoding critical components of the antigen presentation machinery, such as β2-microglobulin (β2M) (Bicknell et al., 1994). The β2M molecule is required for the stable expression of the MHC class I/peptide complex at the cell surface and hence β2M mutations allow irreversible evasion of CTL (Peaper and Cresswell, 2008). However, reduction in tumour cell MHC class I expression can also occur by a second, reversible pathway (Campoli and Ferrone, 2008; Restifo et al., 1993). In this case, reduced expression of antigen presentation pathway components restricts the flow of peptide loaded MHC class I molecules to the cell surface (Restifo et al., 1993). Expression of oncogenic RAS has previously been linked with the reduced expression of antigen presentation pathway components in mouse cells and in human tumour tissue (Seliger et al., 1996, 1998; Atkins et al., 2004) (and reviewed in Pylayeva-Gupta et al. (2011)). These components include the transporter of antigen processing (TAP) and the TAP-associated molecule, tapasin, which are required for efficient delivery of antigenic peptides into the endoplasmic reticulum (ER) (Peaper and Cresswell, 2008). This suggests that targeted inactivation of oncogenic RAS may restore expression of MHC class I molecules to the cell surface and help to boost T cell recognition. However, there are conflicting reports on the association between RAS mutations and antigen presentation in human tumours (Atkins et al., 2004; Delp et al., 2000; Oliva et al., 1990), indicating that functional studies are required. We have analysed the role of mutant RAS oncogenes in regulating the expression of MHC class I molecules and in determining the recognition of RAS mutant tumours by cytotoxic lymphocytes. Our results reveal that oncogenic RAS inhibits the antigen processing pathway in human tumour cells and that these RAS-mediated effects are reversible. However, enhancing the antigen processing pathway by targeting oncogenic RAS in these tumour cells did not alter their susceptibility to NK cells or CTL *in vitro*.

2. Materials and methods

2.1. Culture of tumour cell lines

The inactivation of mutant KRAS in HCT116 (termed H^{Mu} here) and in DLD-1 (D^{Mu}) generating HKe3 (termed H^{WT}) and DKO4 (D^{WT}) respectively, has been previously described (Shirasawa et al., 1993). Mutant and wild-type cells were cultured in DMEM supplemented with 10% foetal calf serum (FCS). The HCT116, SW480 and HT-1080 cells stably transfected with the anti-RAS iDAb were previously generated (Tanaka and Rabbitts, 2010; Tanaka et al., 2007); these cells were cultured in DMEM + 10% FCS supplemented with 1 mg/ml G418, 1 μg/ml puromycin and 0.3 mg/ml hygromycin B to maintain transfected constructs. For induction of iDAb expression, medium was supplemented with 50 μg/ml doxycycline for 48 h prior to analysis by flow cytometry or immunoblotting.

2.2. Protein and mRNA analysis

The following antibodies were used for flow cytometry (antigen, clone-fluorochrome, and supplier); MHC class I, W6/32-PE, Dako; HLA-A2, BB7.2-PE, Serotec; ULBP1, 170818-PE, R&D systems; ULBP2, 165903-PE, R&D systems; MICA/B, 6D4-PE, BD Biosciences; PVR, TX21-FITC, MBL International; Nectin-2, R2.525-PE, BD Biosciences; CD8, SK7-PerCP, BD Biosciences; IFN-γ, 4S-B3-FITC, BD Biosciences; CD107a, H4A3-FITC (or PE), BD Biosciences. The purity of NK cell preparations was determined using the following antibodies; CD56, AF127H3-PE, Miltenyi Biotec; CD3, UCHT1-FITC, BD Biosciences; NKp46, 9E2-APC, BD Biosciences. The MART-1 peptide loaded HLA-A2 pentamer (APC labelled) was purchased from ProImmune, together with an HLA-A2 control pentamer to allow for accurate gating of MART-1 specific T cells. Flow cytometry was performed using a FACS Calibur or LSRII flow cytometer (BD Biosciences) and analysed using FACS Diva or Cellquest Pro (both from BD Biosciences) or FlowJo software (from Treestar). For Western blotting, we used an anti-calnexin polyclonal sera from Cell Signaling Technology and monoclonal antibodies against Tapasin and TAP-1 (from Paul Lehner, University of Cambridge), actin and the FLAG epitope from Sigma–Aldrich. Quantitative RT-PCR analysis of gene expression was performed as in Wilson et al. (2011) using Taqman probe/primer sets from Applied Biosystems/Life Technologies.

2.3. Preparation and functional analysis of NK cells and MART-1 specific T cells

Human NK cells were prepared from blood samples using indirect immunomagnetic separation, using a kit from Miltenyi Biotec, as previously described (Wilson et al., 2011). NK cell purity, as judged by either the $CD56+CD3^{neg}$ or NKp46+ cell surface phenotype was routinely >90%. For IL-2 stimulation, NK cells were cultured for 5–7 days in 50 U/ml IL-2 (R&D systems). NK cell mediated killing of tumour cells and granule exocytosis assays were performed as we have described previously (Wilson et al., 2011; Meade et al., 2009; Holmes et al., 2011), including after siRNA transfection of target cells (Holmes et al., 2011; Scott et al., 2010). T cells restricted to the HLA-A2 restricted MART-1 epitope were generated *in vitro* and assayed as described (West et al., 2009). The tumour target cells were pulsed with 10 ng/ml of either MART-1 peptide (ELAGIGILTV) or the control HER2/neu peptide (ILHNGAYSL) for 30 min prior to co-culture with the CTL. Discarded blood donations (from the Leeds NHS Blood and Transplant Service) were used as a source of HLA-A2+ lymphocytes.

3. Results

3.1. Mutant RAS decreases cell surface expression of MHC class I molecules

The human colorectal cancer cell line HCT116 contains a wild type KRAS allele and a second mutant allele with the oncogenic G13D mutation. We compared the cell surface expression of MHC class I molecules on HCT116 and a derivative (HKe3) in which the mutant KRAS allele has been deleted by homologous recombination (Shirasawa et al., 1993); this cell line retains the wild-type KRAS allele and differs from the parental HCT116 cells only by the absence of mutant KRAS. For simplicity, we refer to this pair of cell lines as H^{Mu} (for HCT116 with mutant KRAS) and H^{WT} (HCT116 with wild-type KRAS). Loss of the oncogenic KRAS allele impairs (but does not halt) growth of these cells both *in vitro* and *in vivo*