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The multikinase inhibitor Sorafenib induces apoptosis and sensitises endometrial cancer cells to TRAIL by different mechanisms

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ABSTRACT

Sorafenib induces apoptosis and enhances Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL)-induced cell killing of tumoural cells. We have investigated the effects of the multikinase inhibitor Sorafenib alone or in combination with TRAIL and agonistic Fas antibodies on endometrial carcinoma cells. We have also focused on the search of the differential molecular mechanisms by which Sorafenib induces cell death and the ones involved in sensitisation to TRAIL. In the present study, we show that Sorafenib induces apoptosis of both endometrial cancer cell lines and human primary cultures and sensitises these cells to TRAIL and agonistic Fas antibodies (aFas)-induced apoptosis. However, Raf/MEK/ERK inhibition by Sorafenib was not responsible for Sorafenib cell death or TRAIL sensitisation of endometrial cancer cells. Sorafenib treatment correlated with a downregulation of both FLICE-Inhibitory Protein (FLIP) and myeloid cell leukaemia-1 (Mcl-1), caused by a proteasomal degradation of both proteins. We evaluated the contribution of FLIP and Mcl-1 downregulation in apoptosis triggered by Sorafenib alone or Sorafenib plus TRAIL. Interestingly, cell death caused by Sorafenib was mediated by downregulation of Mcl-1, but not by FLIP. In contrast, we found that Sorafenib sensitisation of endometrial carcinoma cells to TRAIL- and Fas-induced apoptosis was dependent on FLIP but not on Mcl-1 downregulation. Altogether, we discern the dual mechanisms by which Sorafenib causes cell death from those involved in death receptor sensitisation.

1. Introduction

The Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) and Fas Ligand (FasL) belong to the pro-apoptotic cytokines of the Tumour Necrosis Factor (TNF) superfamily. TRAIL induces apoptosis in many types of cancer with limited cytotoxicity on normal cells[1,2] indicating that it may become a promising anticancer agent.[3,4] TRAIL-based therapies are under current clinical trials in Phases I and II.[5] However, an increasing number of tumoural cell types display resistance

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to TRAIL-mediated cell killing. To circumvent such resistance, combinatorial therapies to inhibit molecular determinants involved in inhibition of apoptosis triggered by TRAIL would be very important. TRAIL triggers apoptosis by binding one of its functional receptors DR4 or DR5.[6] Engagement of DR4 or DR5 receptors leads to the formation of a Death-Inducing Signalling Complex (DISC). The Death Domain (DD) of these receptors recruits Fas Associated DD-containing protein (FADD) which in turn binds pro-caspase-8. After recruitment to the DISC, pro-caspase-8 is activated by autoproteolytic cleavage resulting in the initiation of apoptotic signalling.[7–9] One of the key regulators of apoptosis triggered by either FasL or TRAIL is the FLICE-Inhibitory Protein (FLIP).[10,11] High levels of FLIP are found in many tumoural tissues including endometrial carcinoma. We have previously shown that FLIP plays a critical role in the regulation of sensitivity of endometrial carcinoma cells (ECC) to TRAIL-induced apoptosis. In this previous work, we demonstrated that siRNA mediated inhibition of FLIP sensitised endometrial cancer cells to TRAIL-induced apoptosis.[12] FLIP shares a high degree of homology with caspase-8, and contains two Death Effector Domains (DEDs) and a defective caspase-like domain that lacks proteolytic activity. Thus, high levels of FLIP compete with caspase-8 and displace its binding to FADD, which results in inhibition of apoptosis.

Sorafenib (also known as Bay 43-9006, Nexavar) was initially identified as a Raf-1 inhibitor, but subsequent studies revealed that Sorafenib is a multikinase inhibitor with activity over several kinases, including B-Raf on its wild type and V600 mutated forms; tyrosine kinase receptors such as platelet–derived growth factor, vascular-endothelial growth factors 1 and 2, c-Kit, FLT3 or Ret.[13,14] Sorafenib is currently administered as a chemotherapeutic agent to patients with advanced renal cell carcinoma and there are ongoing clinical trials for melanoma, hepatocellular carcinoma and non-small cell lung cancer.[13,14]

Recent findings show that Sorafenib may enhance TRAIL-induced cell killing on cancer cells.[15–17] The proposed molecular mechanisms by which Sorafenib sensitises cancer cells to TRAIL include downregulation of the myeloid cell leukaemia-1 (Mcl-1),[15] downregulation of Mcl-1 together with FLIP protein levels[17] or a transcriptional reduction of c-IAP2 and Mcl-1.[16] Moreover, the role of Raf kinase activity and its downstream kinases, MAPK/ERK kinase (MEK) and Mitogen-Activated Protein Kinase/Extracellular-Regulated Kinase (MAPK/ERK), as a mechanistic effector of Sorafenib anti-tumour effects is uncertain.

Here, we demonstrated that Sorafenib-induced apoptosis in endometrial carcinoma cell (ECC) lines and sensitised ECC and primary cultures from endometrial carcinoma patients to TRAIL-induced apoptosis. Long-term exposure to Sorafenib alone triggered apoptosis of ECC. However, short-exposure periods to Sorafenib had no killing effects, but dramatically enhanced TRAIL- and agonistic Fas (aFas) antibody-induced apoptosis. Then, we focused on the search of differential molecular mechanisms by which Sorafenib induces cell death and also the ones involved in sensitisation to TRAIL. Sorafenib sensitisation to TRAIL was independent of B-Raf kinase activity or MEK/ERK inhibition. Sorafenib sensitisation correlated with downregulation of FLIP protein levels. Sorafenib mediated FLIP reduction was not caused by transcriptional repression of FLIP but by proteasome degradation, since co-treatment with proteasome inhibitors completely prevented reduction of FLIP levels. Accordingly, FLIP overexpression was sufficient to inhibit Sorafenib sensitisation to TRAIL. In contrast, overexpression of Mcl-1, which effectively prevents apoptosis induced by Sorafenib, did not prevent cells from TRAIL plus Sorafenib-induced apoptosis. Because of the given importance of Sorafenib and TRAIL in cancer therapy, we exposed primary cultures obtained from biopsies of patients with endometrial carcinoma to TRAIL plus Sorafenib. Accordingly with the results obtained in cell lines, Sorafenib sensitised such cancer cells to apoptosis and reduced both Mcl-1 and FLIP levels.

2. Materials and methods

2.1. Reagents, plasmids and antibodies

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT) and monoclonal antibody to Tubulin and anti-Flag M2 were from Sigma (St Louis, MO). Kinase inhibitors PD98059, DRB and apigenin, proteasome inhibitor MG-132, monoclonal antibody to caspase-8 and human recombinant TRAIL were from Calbiochem (La Jolla, CA). Antibody to caspase-9 and cleaved caspase-3 were obtained from Cell Signalling (Beverly, MA). Monoclonal antibody to FLIP (NF6) and aFas antibody were purchased from Alexis Corp (Lausen, Switzerland). Antibody to Mcl-1 was purchased from BDbiosciences (San Jose, CA). Antibody to PARP was from Neomarkers. Anti-B-Raf antibody was from SantaCruz Biotechnology, Inc. (SantaCruz, CA). Peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Amersham-Pharmacia (Uppsala, Sweden). BAY 43-9006 (Sorafenib) was provided by Bayer Pharmaceuticals (New Haven, CT). Bid inhibitor (BI-6C9) was from Sigma.

Lentiviral vector containing Flag-tagged mouse FLIP cDNA was a gift from Dr. Joan Comella (Dept de Bioquímica i Biologia Molecular, Universitat Autonoma de Barcelona, Barcelona). The pCDNA3 vector encoding Mcl-1 cDNA was a generous gift from Dr. Isabel Marzo.

2.2. Cell lines, culture conditions and transfection

The Ishikawa 3-H-12 cell line (IK) was obtained from the American Type Culture Collection (Manassas, VA). KLE cells were a gift from Dr. Palacios (Centro Nacional de Investigaciones Oncológicas, CNIO, Madrid). RL-95/2 and HEC-1A cells were a gift from Dr. Reventos (Hospital Vall d'Hebron, Barcelona). All cell lines were grown in Dulbeco's modified Eagles Medium (DMEM) (Sigma) supplemented with 10% Foetal Bovine Serum (Invitrogen, Inc., Carlsbad, CA, USA), 1 mM HEPES (Sigma), 1 mM sodium pyruvate (Sigma), 2 mM L-glutamine (Sigma) and 1% of penicilin/streptomycin (Sigma) at 37 °C with saturating humidity and 5% CO_2.

When indicated, transfection plasmid constructs were performed by calcium phosphate or Lipofectamine 2000 reagent (Invitrogen) following the manufacturers instructions.