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MDM2 regulates a novel form of incomplete neoplastic transformation of *Theileria parva* infected lymphocytes

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ABSTRACT

Our efforts are concerned with identifying features of incomplete malignant transformation caused by non viral pathogens. *Theileria parva* (*T. parva*) is a tick-transmitted protozoan parasite that can cause a fatal lymphoproliferative disease in cattle. The *T. parva*-infected lymphocytes display a transformed phenotype and proliferate in culture media like the other tumor cells, however those cells will return to normal after antiprotozoal treatment reflecting the incomplete nature of transformation. To identify signaling pathways involved in this form of transformation of *T. parva*-infected cells, we screened a library of anticancer compounds. Among these, TIBC, a specific inhibitor of MDM2, markedly inhibited proliferation of *T. parva*-infected lymphocytes and promoted apoptosis. Therefore we analyzed MDM2 function in *T. parva*-infected cells. Several *T. parva*-infected cell lines showed increased expression level of MDM2 with alternatively spliced isoforms compared to the lymphoma cells or ConA blasts. In addition, buparvaquone affected MDM2 expression in *T. parva* transformed cells. Moreover, p53 protein accumulation and function were impaired in *T. parva*-infected cells after cisplatin induced DNA damage despite the increased p53 transcription level. Finally, the treatment of *T. parva*-infected cells with boronic-chalcone derivatives TIBC restored p53 protein accumulation and induced Bax expression. These results suggest that the overexpression of MDM2 is closely linked to the inhibition of p53-dependent apoptosis of *T. parva*-infected lymphocytes. Aberrant expression of host lymphocyte MDM2 induced by cytoplasmic existence of *T. parva*, directly and/or indirectly, is associated with aspects of this type of transformation of *T. parva*-infected lymphocytes. This form of transformation shares features of oncogene induced malignant phenotype acquisition.

Introduction

Members of the genus *Theileria* are intracellular apicomplexan protozoan parasites transmitted by ticks. Among several *Theileria* species, *T. parva* is highly pathogenic for cattle and cause fatal lymphoproliferative diseases known as East Coast fever (Brown et al., 1973; Irvin et al., 1975; Lawrence and Irvin, 1994). The schizont stage of *T. parva* resides within leukocytes and has direct contact with the host-cell cytoplasm and matrix. *T. parva* is among several parasites that inhibit host-cell apoptosis pathways to ensure their intracellular survival (Heussler et al., 2001b). The parasites have the unique ability to transform host lymphocytes and to synchronize their division with that of the host cell (Hulliger et al., 1964; von Schubert et al., 2010), ensuring that infection is maintained in daughter lymphocytes. Parasite multiplication is dependent on host-cell proliferation. The transformation and immortalization of *T. parva*-infected cells is very reminiscent of tumor cells, a major difference being that *T. parva*-induced transformation is reversible, as leukocytes return to a resting phenotype upon elimination of the parasite by the anti-parasitic agent buparvaquone. This suggests that transformation is not dependent on defined genomic changes in the host cell (Dobbelaere and Heussler, 1999). However, the underlying mechanisms by which *T. parva*-induces incomplete transformation have not been defined.

p53 acts as a tumor suppressor in mammalian cells as reviewed in (Gottlieb and Oren, 1996). Under normal conditions, p53 is maintained at low levels due to its rapid turnover and degradation by proteasomes (Vogelstein et al., 2000). In response to diverse types of stress, p53 can accumulate and can influence cell cycle progression. The mouse double minute 2 (*mdm2*) gene was originally identified as an amplified gene on the double-minute chromosome in a spontaneously transformed

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mouse BALB/c 3T3 cell line (Cahilly-Snyder et al., 1987). The MDM2 protein acts as a negative regulator of p53 through two known mechanisms: First, MDM2 binds p53 through its NH2-terminus, resulting in inhibition of its transcriptional activity (Momand et al., 1992), and second, the COOH-terminus of MDM2 serves as an E3 ubiquitin ligase targeting p53 for proteasomal degradation (Haupt et al., 1997). MDM2 can exhibit oncogenic activity when overexpressed in cells (Fakharzadeh et al., 1991), and gene amplification and overexpression of MDM2 protein are found in about 10% of human tumors (Toledo and Wahl, 2006). In addition, many spliced isoforms of MDM2 are observed in human tumors, and the cDNA coding some of these spliced isoforms is also capable of transforming cells (Sigalas et al., 1996). Thus, both overexpression and alternative splicing of MDM2 are thought to contribute to its oncogenic function.

In this study, we screened *T. parva*-infected lymphocytes for susceptibility to a library of compounds with known anti-mitotic activity in an attempt to identify signaling pathways involved in causing parasite-induced unlimited cell proliferation.

TIBC is an anticancer agent that has been suggested to affect the MDM2/p53 protein complex to selectively inhibit the growth of MDM2-overexpressing tumors (Kumar et al., 2003). The anti-tumor effects of TIBC, which have been confirmed in cancers *in vitro* and *in vivo* (Sasayama et al., 2007), has shown some promise for chemotherapeutic use, especially against tumors in which MDM2 overexpression occurs in the absence of genomic mutations in p53. In this study, we found that the TIBC specifically and efficiently inhibited proliferation of *T. parva*-infected lymphocytes, leading to apoptosis. We analyzed the MDM2 and p53 status of several *T. parva*-infected cell lines with respect to mRNA and/or protein expression levels to elucidate the fundamental mechanisms of incomplete transformation of lymphocyte induced by intracellular infection of *T. parva*.

Materials and methods

Cell culture

Cell lines infected with the Muguga or Marikebuni stocks of *T. parva* (Brown and Logan, 1986) were derived by *in vitro* infection of peripheral blood mononuclear cells (PBMC) or in one case (G6TpM, provided by Dr. R. Bishop, International Livestock Research Institute, Nairobi, Kenya) a cloned bovine T lymphoblastoid cell line, with sporozoites. Two of the lines (951-I38TPMa and 951-E43TPMa), derived from the same animal, had been cloned by limiting dilution and contained genotypically distinct parasite clones (Katzer et al., 2006). The designated numbers (592, 011, 641, or 951) indicate animal numbers of healthy Holstein cattle from which blood was collected. Control uninfected activated lymphocytes were prepared by stimulation of PBMCs from the same animals (011, 641, and 951) with concanavalin A (ConA; 0.5 μg/ml). *T. parva*-infected cell lines were maintained in RPMI-1640 culture medium containing 10% heat-inactivated fetal bovine serum (FBS), 50 μM 2-mercaptoethanol, 50 units/ml penicillin, and 50 μg/ml streptomycin. For maintenance of ConA-treated lines, 10 U/ml recombinant mouse IL-2 (R&D Systems) was also added to the medium. Madin-Darbey bovine kidney (MDBK) cells and a bovine leukemia transformed cell line BTL-26 (kindly provided by Dr. N. Ishiguro, Gifu University, Japan) (Komori et al., 1996) were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin. To eliminate *T. parva* schizonts from the infected cells (011TPM), cells were cultured with 100 ng/ml buparvaquone (Butalex; Essex Animal Health) in the presence of 100 U/ml recombinant mouse IL-2 for 2 or 5 days. Studies involving the use of animals were conducted under a Project Licence issued by the UK Home Office and all experimental procedures were approved by the University of Edinburgh Animal Ethics Committee. The number of the Home Office Licence under which the work was carried out is 60/3736.

Screening of compounds for inhibition of T. parva-infected cell proliferation

Cell proliferation assays were performed to examine whether anticancer compounds affected growth of *T. parva*-infected lymphocytes. The SCADS inhibitor kits I and II, consisting of 190 kinds of inhibitors, were kindly provided by the Screening Committee of Anticancer Drugs supported by Grant-in-Aid for Scientific Research on Priority Area "Cancer" from The Ministry of Education, Culture, Sports, Science and Technology, Japan. These compounds were added to 5×10^4 *T. parva*-infected G6TpM cells or ConA-stimulated lymphocytes in 96-well plates at a concentration of 10 μM, then proliferation was quantified after 24 h incubation using a BrdU proliferation ELISA kit (Roche) according to the manufacturer's instructions.

Effects of TIBC on cell death and p53 expression in T. parva-infected lymphocytes

G6TpM cells (1.5×10^5), BTL-26 cells and MDBK cells were treated with TIBC (Merck-Calbiochem) for 50 h in 24-well plates. For the detection of apoptotic cells, cells were stained with propidium iodide (PI) and Allophycocyanin-conjugated annexin V (BD Biosciences), and analyzed by flow cytometry on a FACSCanto cell analyzer (BD Biosciences). 011TPM, 951-E43TPMa, and 951ConA cells were similarly treated with TIBC in 24-well plates, and 2×10^4 cells from each treatment group were then transferred to a 96-well white plate. To indicate apoptosis, the activity of caspase 3 and 7 was quantified using the Caspase-Glo 3/7 kit (Promega) according to the manufacturer's instructions. Luminescence was measured with a luminometer (Victor; PerkinElmr). For the induction of DNA damage, 5×10^5 G6TpM, BTL-26, and MDBK cells were treated with cisplatin (Merck-Calbiochem) for 24 h at 0, 5, 20 or 50 μM in 8-well plates, and subjected to Western blot analysis as described below. For detection of p53 protein or the transcript of its downstream target Bax, 5×10^5 G6TpM was treated with TIBC for 50 h at 0, 5, 10 or 25 μM in 8-well plates. Cells were harvested, and Western blot analysis was performed as described below.

RT-PCR for detection of spliced isoforms of mdm2

Total RNA was isolated from cells by the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen). cDNAs were then synthesized from total RNA in the presence of oligo(dT) primers using a Ready-To-Go You-Prime First-Strand Beads kit (GE Healthcare) in accordance with the manufacturer's instructions. The resulting cDNAs were used in nested PCR to detect *mdm2*. Nested PCR primers were designed based on the bovine *mdm2* sequence, mdm2F1 (5′-AACTGGGGAGCCTCGGGGGA-3′) and mdm2R1 (5′-GGTATTATC TTGCTTTGATACACCT-3′) for the first amplification, and mdm2F2 (5′-GTTAGTGAGCATCAGGCAAATGTG-3′) and mdm2R2 (5-TTACAGG TAGTTCAACTAGGGG-3′) for the second amplification. Each PCR was performed under the following conditions: 95 °C for 1 min, 25 amplification cycles (95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min), and a final extension step (72 °C for 7 min). As an internal control, *gapdh* amplification was performed with BoGAPDH-F (5′-TTCAAC GGCACAGTCAAGG-3′) and BoGAPDH-R (5′-ACATACTCAGCACC AGCATCAC-3′), using the conditions described above, except for the annealing temperature, which was 57 °C. Amplification of the *mdm2b* isoform was also performed using specific primers (BoMBforward: 5′-AAGAGACCCTGGACTATTGGAAGTG-3′ and BoMBreverse: 5′-TGCCA TTGAACCTTGTGTGATTTG-3′) after a first amplification with mdm2F1 and mdm2R1, as previously described (Steinman et al., 2004). For *mdm2b* isoform detection, a 30 amplification cycle was used while the rest of the PCR protocol remained the same as above. The PCR products were resolved on 1.2% agarose gels, isolated, cloned into the pGEM-T Easy Vector system (Promega), and sequenced in an ABI Prism 3700 Analyzer (Applied Biosystems).