Abstract. The aim of this study was to evaluate the additive effect of valproic acid (VPA) to γδ T-cell cytotoxicity against bladder cancer cells. Materials and Methods: Human bladder cancer cell lines TCCSUP and 253J were treated with VPA and mRNA expression of natural killer group 2D (NKG2D) ligands was determined. The antitumour effect of expanded γδ T-cells against zoledronic acid (ZOL) and VPA pre-treated cancer cells was subsequently determined. Results: VPA increased mRNA expression of NKG2D ligands on both cancer cell types. A blocking study revealed that 253J cells were recognised through NKG2D, while TCCSUP cells were mainly recognised through γδ T-cell receptor. VPA pre-treatment increased sensitivity to cytolysis by γδ T-cells for both cancer cell types, whereas ZOL pre-treatment was only effective against TCCSUP. Conclusion: Induction of NKG2D ligands by VPA increased the susceptibility of cancer cells that are recognised by NKG2D to cytolysis by γδ T-cells.

Superficial bladder carcinomas represent 70% of all bladder carcinomas and are managed with transurethral surgical treatment followed by intravesical instillation of chemotherapeutic agents or Bacillus Calmette-Guerin (BCG) treatment (1). BCG, which exerts antitumour effects through the local immune response, is considered the most effective agent, particularly in carcinoma in situ (2). Other immunotherapeutic approaches, including vaccine therapy, have been considered, but durable responses are rare (3). For this reason, MHC class I down-regulation leads to tumour evasion from αβ T-cells (cytotoxic T lymphocytes) particularly in high-grade cancer (4). In contrast, innate immune surveillance includes γδ T-cells, which are only a small subset (1-10%) of peripheral blood T-cells. Unlike conventional αβ T-cells, γδ T-cells recognise antigens in a MHC-unrestricted manner, suggesting their potential as a novel immunotherapeutic approach against cancer cells. γδ T-Cells recognise phosphoantigens (e.g., isopentenyl pyrophosphate [IPP]) in a T-cell receptor (TCR)-dependent manner (5).

Zoledronic acid (ZOL) is widely used to treat benign and malignant bone disease. ZOL inhibits farnesyl pyrophosphate synthase, a key enzyme in the mevalonate pathway (6). ZOL treatment results in accumulation of upstream metabolites (e.g., IPP), thereby inducing expansion of γδ T-cells in vitro and in vivo. Thus, ZOL pre-treatment increases the susceptibility of various cancer cells to cytolysis by γδ T-cells (7). In addition to TCR-dependent recognition, γδ T-cells recognise infected or cancer cells via interaction between the activating receptor natural killer group 2D (NKG2D), expressed on γδ T-cells, and NKG2D ligands, expressed on cancer cells (e.g., MHC class I chain-related proteins A and B [MICA/B]) (5).

Valproic acid (VPA), the use of which is well-established in the treatment for epilepsy and bipolar disorder, has been shown to inhibit histone deacetylase (HDAC). Recent reports have demonstrated that HDAC inhibition enhances natural killer (NK) cell-mediated cytotoxicity against cancer cells through up-regulation of NKG2D ligands (8, 9). However, therapeutic induction of NKG2D ligands for γδ T-cell lysis has not yet been achieved. Therefore, the purpose of this study was to investigate the additive effect of VPA to γδ T-cell cytotoxicity against human bladder cancer cells.
Materials and Methods

Reagents and cancer cell lines. ZOL was obtained from Toronto Research Chemicals Incorporation (Toronto, Canada). High-grade human bladder cancer cell lines TCCSUP and 253J were obtained from the American Type Culture Collection (Manassas, VA, USA).

Preparation of γδ T-cells. After informed consent was obtained from healthy volunteers, 20 ml venous blood was withdrawn. Human peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with Ficoll-Paque (GE Healthcare, Uppsala, Sweden). PBMCs (1×10⁶ cells/ml) were seeded into 6-well flat-bottom plates (Corning, Inc., NY, USA) and stimulated with 1 μM ZOL and 100 U/ml IL-2 on day 0. The culture medium consisted of RPMI-1640 supplemented with 10% pooled human AB serum (Cosmo Bio, Tokyo, Japan). IL-2 (100 U/ml) was added to the culture and three quarters of the medium was replaced with AlyS505N medium (Cell Science & Technology Institute, Miyagi, Japan) supplemented with 2 mM L-glutamine (Cosmo Bio) every three days. After ten days of culture, expanded γδ T-cells were purified by magnetic-activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). Subsequent flow cytometric analysis confirmed that more than 98% of purified cells expressed γδ TCR (data not shown).

Real-time quantitative PCR. Both bladder cancer cell lines were incubated with or without 0.25 mM VPA for 72 h. Total cellular RNA was isolated with a TaKaRa RNA extraction kit (Takara Bio, Shiga, Japan) and cDNA was synthesized with a reverse transcription kit (TaKaRa RNA PCR Kit Ver. 3.0; Takara Bio) following the manufacturer’s protocol. Polymerase chain reaction (PCR) was performed with sequence-specific primers and TaqMan reagents in the StepOnePlus Real-Time PCR System v2.0 (Applied Biosystems, Carlsbad, CA, USA). Table I shows nucleotide sequences of TaqMan primers and probes for MICA/B and GAPDH (data not shown).

Blocking study. γδ T-Cells express two activating receptors, γδ TCR and NKG2D. Blocking antibodies were used to determine the predominant receptor for each cancer cell line. γδ T-Cells were seeded in 96-well round-bottom plates (Corning) and incubated for 1 h with 10 μg/ml anti-pan γδ TCR blocking monoclonal antibody (Biodisign International, Saco, ME, USA), anti-NKG2D blocking monoclonal antibody (R&D Systems, Minneapolis, MN, USA), or isotype control mouse IgG1 (BioLegend, San Diego, CA, USA). Cancer cells were then added at an effector:target (E:T) ratio of 10:1. The predominant receptor was determined by inhibition of the γδ T-cell antitumour effect as assessed by the alamarBlue assay.

Cytotoxicity assay. The antitumour effect of γδ T-cells against untreated or pre-treated bladder cancer cells was assessed by the alamarBlue assay. Briefly, actively proliferating cancer cells incubated with 0.25 mM VPA for 72 h or 5 μM ZOL for 12 h were seeded in 96-well round-bottom plates (5×10³ cells/well). γδ T-Cells were added at various E:T ratios. After 4 h, 20 μl alamarBlue (Invitrogen Corp., Carlsbad, CA, USA) was added, and the plate was read with a SpectraMax® M2e spectrophotometer (excitation, 530-560 nm; emission, 590 nm) using SoftMax Pro 5.2 software (all from Molecular Devices, Inc., Sunnyvale, CA, USA). Viability of the cancer cells at each E:T ratio was calculated according to the formula: Viability (%)=100 × (γδ T-cells + cancer cells per well – γδ T-cell controls per well)/cancer cell controls per well.

Statistical analysis. Results were compared using analysis of variance (ANOVA) and Bonferroni correction. Statistical analysis was performed using Excel (Microsoft, Inc., Redmont, WA, USA) with ystat2006 (Igakutosho Shuppan Ltd, Tokyo, Japan). P<0.05 was considered statistically significant.

Results

Relative quantification of mRNA expression of NKG2D ligands after VPA treatment. VPA significantly increased MICB mRNA expression 17.7-fold of TCCSUP cells, but not MICA mRNA expression (Figure 1A). Similarly, mRNA expression of MICA was significantly increased 1.8-fold; MICB mRNA expression was slightly decreased on 253J cells (Figure 1B).
Predominance of each receptor in TCCSUP and 253J cells. As shown in Figure 2A, the γδ T-cell antitumour effect against TCCSUP cells was significantly inhibited by anti-NKG2D blocking monoclonal antibody \( (p=0.013) \) and was significantly inhibited by anti-γδ TCR blocking monoclonal antibody \( (p>0.05) \). In contrast, the γδ T-cell antitumour effect against 253J cells was slightly inhibited by anti-γδ TCR blocking monoclonal antibody \( (p>0.05) \), but was significantly inhibited by anti-NKG2D blocking monoclonal antibody \( (p=0.008) \) (Figure 2B).

Functional relevance of altered mRNA expression of NKG2D ligands for γδ T-cell cytolysis. At a 40:1 E:T ratio, γδ T-cells lysed up to 51% of untreated TCCSUP cells; VPA pre-treatment significantly increased TCCSUP cell sensitivity to cytolysis by γδ T-cells to 65% \( (p=0.042) \) and ZOL pre-treatment significantly increased sensitivity to cytolysis to 73% \( (p=0.014) \) (Figure 3A). The antitumour effect of γδ T-cells against ZOL pre-treated TCCSUP cells was increased compared with untreated and VPA pre-treated cells.

Similarly, at a 40:1 E:T ratio, γδ T-cells lysed up to 61% of untreated 253J cells; VPA pre-treatment significantly increased sensitivity to cytolysis to 88% \( (p=0.033) \), and ZOL pre-treatment increased sensitivity to cytolysis to 72% \( (p>0.05) \) (Figure 3B). The antitumour effect of γδ T-cells against VPA pre-treated 253J cells was higher than the effect against untreated and ZOL pre-treated cells. These findings were consistent with the results of the blocking study.

Discussion

There are two strategies for cancer immunotherapy using γδ T-cells. The first strategy is co-administration of ZOL and IL-2 to expand γδ T-cells \textit{in vivo} (10). However, repeated administration of ZOL has been reported to result in lower response (anergy) compared with initial treatment (11). The second strategy is adoptive transfer of \textit{ex vivo} expanded γδ T-cells (12, 13). However, in a recent clinical trial, severe dose-limiting toxicity was observed with the dose of \( 8\times10^9 \) γδ T-cells against renal cell carcinoma (14). A complete
tumour response has not been achieved with a low dose of γδ T-cells; therefore, strategies combining chemotherapy (15) and antiangiogenic agents (12) or monoclonal antibodies (16) have been investigated. In addition, up-regulation of NKG2D ligand after HDAC inhibitor treatment has been reported to enhance NK cell-mediated cytotoxicity against cancer cells. Therefore, the additive effect of HDAC inhibitor VPA on in vitro γδ T-cell cytotoxicity was evaluated in this study.

In addition, two activating receptors expressed by γδ T-cells are γδ TCR and NKG2D. Wrober et al. reported that the predominant γδ TCR differs depending on the cancer cell line (17). Therefore, the receptor predominance against human bladder cancer cells was determined using blocking monoclonal antibodies.

VPA generally increased mRNA expression of MICA/B on both bladder cancer cells. Although precise mechanisms for this increase with VPA treatment are unclear, HDAC inhibitors appear to switch the nucleosome structure to an open state to allow access of transcription factors to NKG2D ligands, resulting in gene activation (8, 18). Induction of NKG2D ligand expression by VPA increased the susceptibility of 253J cells, which are predominantly recognised by NKG2D, to cytolysis by γδ T-cells, whereas ZOL increased the susceptibility of TCCSUP cells, which are predominantly recognized by γδ TCR, to cytolysis. VPA also enhanced cytotoxicity in TCCSUP cells, indicating that TCCSUP cells are primarily recognised through γδ TCR and partially recognised through NKG2D. However, ZOL pre-treatment exerted only a mild effect in 253J cells mainly recognised by NKG2D.

In contrast, MICB mRNA expression was slightly decreased on 253J cells after VPA treatment. NKG2D
ligands are shed and released from the cancer cell surface; the down-regulated surface expression of NKG2D leads to immune evasion (19), especially in high-grade carcinomas (20). Therefore, soluble MICB may be increased by VPA treatment in 253J cells. Further investigations are necessary to determine soluble NKG2D ligands after VPA treatment.

Taken together, the findings of the present study demonstrated that VPA increases the susceptibility of cancer cells, which are predominantly recognised by NKG2D, to cytolysis by γδ T-cells. Thus, the results of this study may be an indicator of a novel adoptive immunotherapy against bladder carcinoma.

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References