

Combinatorial Cytotoxicity of Gemcitabine and Cytokine-activated Killer Cells in Hepatocellular Carcinoma *via* the NKG2D-MICA/B System

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Abstract. Aim: Natural-killer group 2, member D (NKG2D) is an activating receptor on natural killer cells and activated T-cells, designated cytokine-activated killer (CAK) cells here. The MHC class I chain-related A and B (MICA and MICB, respectively) are ligands of NKG2D and are expressed on various human tumor cells, including hepatocellular carcinoma (HCC) cells. Here, we investigate whether gemcitabine, a chemotherapeutic agent, affects MICA/B expression in HCC. Materials and Methods: We used ELISA, RT-PCR and adherent target detachment assays to determine expression of MICA/B in HepG2 HCC cells and the level of cellular cytotoxicity generated by treatment with gemcitabine and/or CAK cells. Results: Surface expression of MICA/B was evident after gemcitabine treatment, and MICB-specific mRNA was up-regulated. Pre-treatment with gemcitabine and subsequent exposure to CAK cells induced greater cytotoxicity than either treatment alone. Inclusion of soluble MICB significantly reduced cytotoxicity. Conclusion: Gemcitabine induced MICA/B expression in HepG2 cells, resulting in synergistic enhancement of the cytotoxic effects of NKG2D-high CAK cells. The combination of gemcitabine and CAK cells may have clinical therapeutic significance for HCC.

Natural killer (NK) cells, $\gamma\delta^+$ T-cells, and activated CD8⁺ T-cells are important effectors in the immune response to

tumors, and have been used in cell transfer therapy for various types of cancer (1-5). A number of inhibitory and activating receptors on the surface of these cells tightly regulate their interaction with target cell ligands (1, 2). In particular, the strength of the antitumor immune response appears to be critically dependent on surface levels of one activating receptor, natural-killer group 2, member D (NKG2D) (5, 6). Correspondingly, expression of NKG2D ligands on target cells is a requirement for effective tumor immunosurveillance and the elimination of cancer cells. MHC class I-related chain (MIC) A and B (MICA and MICB, respectively) are polymorphic transmembrane glycoproteins and are ligands for NKG2D. Expression of MICA/B can be induced by various cellular and environmental stimuli, including heat shock, virus infection, and DNA damage-inducing agents (7, 8).

Gemcitabine, a potent chemotherapeutic agent for various cancers including hepatocellular carcinoma, has recently been proposed to have immunomodulatory effects (9, 10). Nowak *et al.* showed that gemcitabine suppressed IgG antibody production, but did not block lymphocyte recall response and was not detrimental to specific antitumor immunity (11). It has also been found that gemcitabine does not diminish cellular immunology, suggesting that cellular and molecular therapies could therefore be used in parallel to greater effect than chemotherapy alone (10, 11). Indeed, Bauer *et al.* reported that a combination of a dendritic cell vaccine and gemcitabine was effective in a pancreatic cancer model (12). Thus, gemcitabine appears to be a good candidate drug for combination with immunotherapy.

Here, we investigated the effect of gemcitabine on the expression of MICA/B RNA and protein in the HepG2 tumor-cell line, and the susceptibility of these cells to cytotoxicity exerted by activated NK cells and activated T-cells, hereafter defined as cytokine-activated killer cells (CAK).

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Key Words: Gemcitabine, NKG2D, MICA, MICB, HCC, immunotherapy.

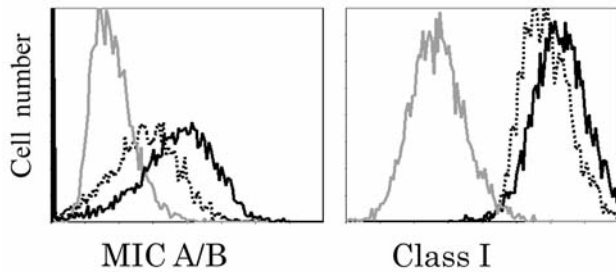


Figure 1. Gemcitabine induces MICA/B and MHC class I expression. Cells were treated with gemcitabine at 10 $\mu\text{g/ml}$ for 48 h, and MICA/B expression was quantified by flow cytometry. Left panel: Representative histogram of flow cytometric analysis (MICA/B). Gemcitabine-treated cells (solid line) were compared to untreated cells (dashed line). The grey line indicates isotype control antibody staining. Right panel: As for the left panel but showing expression of MHC class I.

Materials and Methods

mAb reagents and flow cytometry. Surface markers were labeled by direct or indirect immunofluorescence using monoclonal antibodies (CD3-FITC, CD16-FITC, CD56-PE: Immunotech, Beckman Coulter, France; NKG2D-PE: R & D Systems, USA). Cell-surface expression of MICA/B was detected using flow cytometry. Detached cells were washed twice in cold phosphate-buffered saline (PBS) and stained with phycoerythrin (PE)-labeled mouse anti-human MICA/B antibody (R & D Systems) for 60 min at 4°C. Fluorescence was detected using an FC500 flow cytometer (Beckman Coulter, France) and expressed as relative mean fluorescence intensity (MFI) or percentage above baseline.

Culture of tumor cells. HepG2 cells were obtained from RIKEN Cell Bank (Osaka, Japan) and cultured in RPMI medium supplemented with 5% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, USA) and 1% penicillin/streptomycin (Meiji Seika, Japan). Viable cells were counted using Cell Counting Kit-8 (Dojindo, Japan).

Generation of ex vivo expanded CAKs. CAKs were induced from peripheral blood mononuclear cells (PBMCs) obtained from four HCC patients with written informed consent. PBMCs were incubated with a high concentration of recombinant IL-2 (rIL-2, 2000 U/ml; Chiron, USA) and 5 $\mu\text{g/ml}$ OKT3 (Orthoclone® OKT3; Janssen Pharmaceutical, Tokyo, Japan) and expanded for 10–14 days to obtain sufficient numbers of CAKs.

Cytotoxicity assay. We modified an adherent target detachment (ATD) assay described previously (13, 14) to measure the cytotoxicity of CAKs. Target cells (5,000 per well) were seeded in a 96-well flat bottom plate and incubated for 24 h to allow adherence. Gemcitabine (0–10 $\mu\text{g/ml}$) was then added to wells and cells were incubated for a further 24 h prior to addition of effector cells at an effector:target cell (E:T) ratio of 10:1 or 20:1. Target and effector cells were then incubated for 4 h. Dead target cells detached from the culture surface and were recovered by washing, together with the added effector cells. To quantify viable adherent cells, WST-8 reagent solution (from the Cell Counting Kit-8) was added

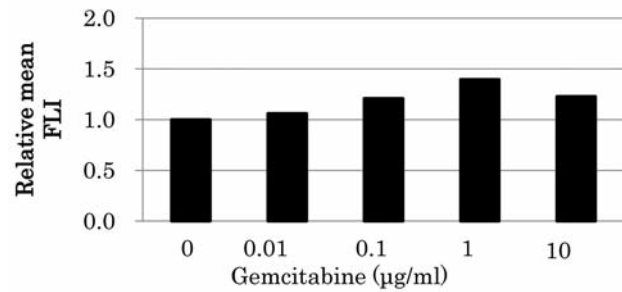


Figure 2. MICA/B staining of HepG2 cells. Cells were treated with the indicated amount of gemcitabine for 48 h. Representative normalized mean fluorescence (MFI of gemcitabine-treated cells/MFI of control cells) data from three different experiments are shown.

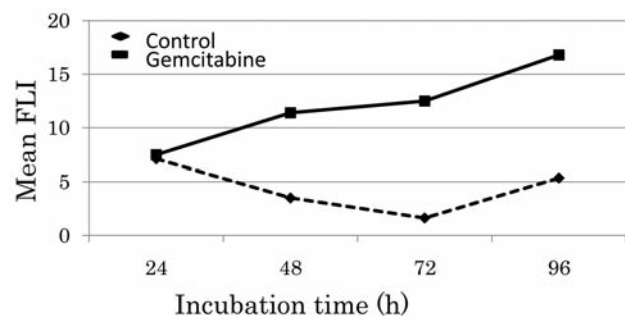


Figure 3. Changes in MICA/B expression in HepG2 cells after exposure to gemcitabine. HepG2 cells were treated for the indicated time with or without gemcitabine (10 $\mu\text{g/ml}$), then stained with anti-MICA/B mAb and analyzed for mean fluorescence.

to the washed wells and they were incubated for 1 h at 37°C. The absorbance at 450 nm was then measured using a microplate reader (ImmunoMini NJ-2300; Nalge Nunc International). Detached cells were stained with 7-amino-actinomycin D (7-ADD; Beckman Coulter) to confirm that detached tumor cells were indeed non-viable. In some experiments, soluble recombinant MICB (American Research Products, USA) was added at different concentrations to HepG2 cells followed by 4 h incubation prior to adding CAK cells.

Quantitative real-time PCR. To perform real-time reverse transcription-PCR, total RNA was isolated using High Pure RNA Isolation kit (Roche Diagnostic GmbH, Mannheim, Germany) followed by DNase digestion and reverse transcription using a Transcriptor High Fidelity cDNA Synthesis kit (Roche). Primer sequences were as follows: *MICB* 5'-ACCTTGCTATGAAGGTCACA3' (forward) and CCCTCTGAGACGTCGCTGCA3' (reverse), *GAPDH*: 5'-ATGACATCAAGAAGGTGGT3' (forward) and 5'-CATACCAGGAAATYGAGCTTG3' (reverse). The resulting cDNA was amplified with *GAPDH*- and *MICB*-specific primers (35 cycles: denaturation at 95°C for 10 s, annealing at 55°C for 5 s, and extension at 72°C for 5 s) using SYBR Green chemistry on the Light Cycler (Roche Diagnostics GmbH, Mannheim, Germany). Relative *MICB* mRNA expression was calculated by normalization against *GAPDH* expression.

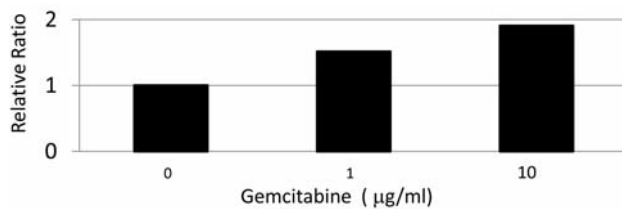


Figure 4. Real-time reverse transcription-PCR of *MICB* mRNA expression levels. Data are expressed as the fold increase of mRNA expression in gemcitabine-treated HepG2 cells relative to untreated cells.

Determination of MICB levels in the culture medium. Secretion of MICB by HepG2 cells cultured under various conditions was assessed using the human MICB DuoSet ELISA kit (R & D Systems) following the manufacturer's instructions. Soluble MICB (sMICB) was detected using a sandwich ELISA procedure. Plates were coated with an anti-MICB mAb (5 µg/ml; R&D Systems) and blocked with 2% BSA in PBS. Tissue culture supernatant was added for 1 h at 37°C. Bound MICB protein was detected using biotinylated goat anti-MICB (R&D Systems) followed by streptavidin horse radish peroxidase (HRP) and developed using the peroxidase substrate system. Absorbance was measured at 450 nm. All samples were analyzed in triplicate.

Statistical analysis. All data are expressed as the mean±standard error of the mean. Differences between groups were assessed for statistical significance using the Mann-Whitney test or paired Student's *t*-test depending on the distribution of the data. $P < 0.05$ was taken to indicate statistical significance.

Results

Gemcitabine up-regulates MICA/B expression in HepG2 cells. We first examined the expression levels of MICB protein on the cell surface in the presence of gemcitabine using flow cytometric analysis with an antibody that recognizes both MICA and MICB. HepG2 cells were cultured with gemcitabine (0.001, 0.01, 0.1, 1 or 10 µg/ml) for 24, 48, 72 or 92 h prior to determination of MICA/B expression. Treatment with gemcitabine markedly augmented MICA/B protein expression on the surface of HepG2 cells as shown in Figure 1. Modulation of MICA/B expression on HepG2 cells was concentration-dependent, with appreciable up-regulation shown in cells treated with gemcitabine concentrations of 0.01 µg/ml or more for 48 h (Figure 2). A time-course study of HepG2 cells treated with 10 µg/ml gemcitabine revealed that MICA/B expression increased time-dependently, but in untreated HepG2 cells, expression decreased dramatically before recovering slightly at 96 h (Figure 3).

We next determined the effects of gemcitabine on the mRNA expression of *MICB* in HepG2 cells. Quantitative real-time PCR analysis revealed that *MICB* transcripts increased after incubation of cells with gemcitabine for 24 h (Figure 4).

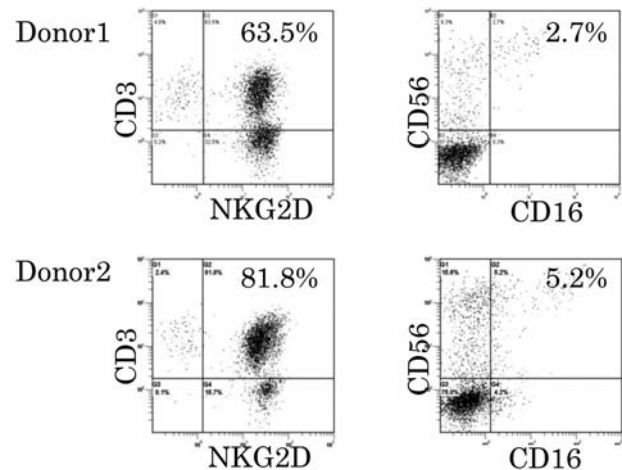


Figure 5. NKG2D expression in activated lymphocytes. Activated and expanded human peripheral blood mononuclear cells (PBMCs) at day 14 of culture were treated with high dose IL-2 (2,000 U/ml) and OKT3 (5 µg/ml), washed and subjected to FACS analysis. Lymphocytes were gated and CD3/NKG2D expression analyzed for this population of lymphocytes.

Increased MICA/B expression following gemcitabine treatment enhances the susceptibility of HepG2 cells to CAK lysis. We next evaluated the effect of gemcitabine treatment on the sensitivity of HepG2 cells to the actions of killer cells by measuring the cytotoxicity of CAK cells against HepG2 cells that had been pretreated with gemcitabine, using the ATD assay described in the Materials and Methods. To determine whether altered levels of MICA and MICB on tumor cells aid the recognition of HepG2 cells by CAK cells, we measured NKG2D expression in effector CAK cells from two different donors (Figure 5). When subjected to cytotoxicity testing, we found that pretreatment with gemcitabine (0.01 or 0.1 µg/ml) significantly increased the susceptibility of the HepG2 cells to cytotoxicity by CAK cells for both donor 1 and donor 2 cells (Figure 6). Furthermore, this synergistic effect of the gemcitabine plus CAK therapy was evident at all time points studied (Figure 7). Morphological changes in HepG2 cells after treatment with gemcitabine, CAK cells or both were also evaluated, and representative images are shown in Figure 8. In both treatment (Figure 8 lower right panel), it can be seen that almost HepG2 cells have lysed.

Effects of gemcitabine on MICB shedding. Soluble NKG2D ligands have been shown to inhibit NKG2D-mediated antitumor cell activity. There is considerable evidence that shedding of MIC ligands in a soluble form represents a mechanism of tumor escape from NKG2D-mediated immune surveillance, and that engagement of soluble MIC with NKG2D has been shown to down-regulate NKG2D expression on CD8⁺ T-cells and to suppress T-cell activation (6).

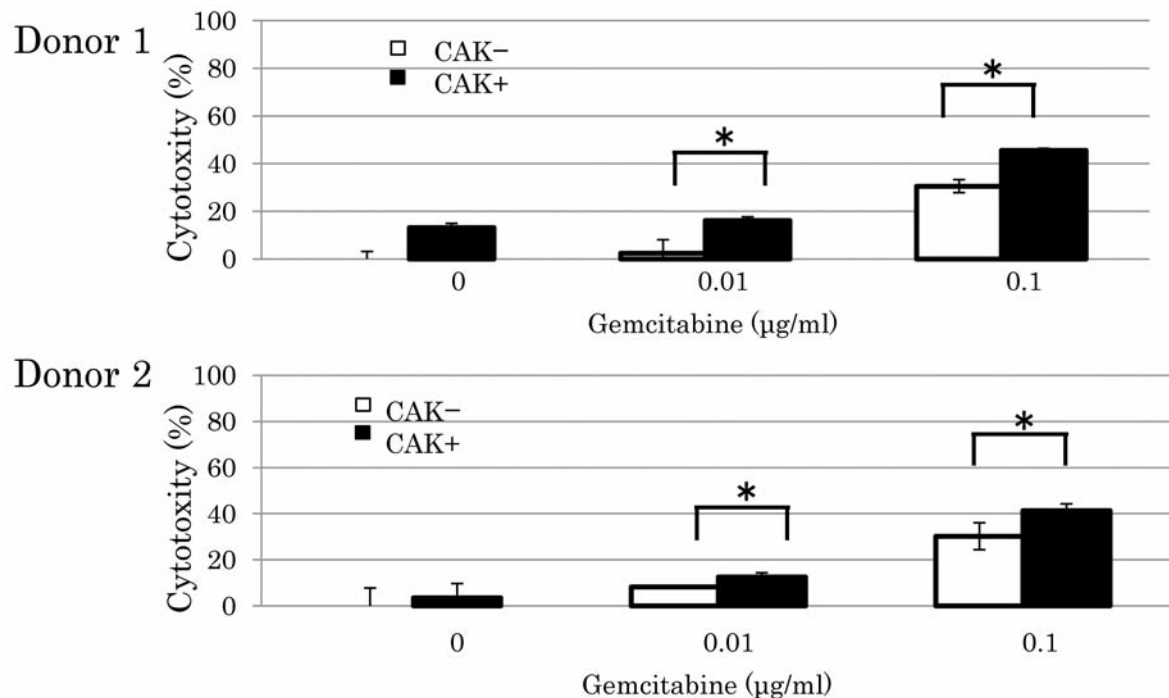


Figure 6. Cytotoxic activity of CAK cells against HepG2 cells with and without gemcitabine. Adherent target detachment (ATD) assay was used as described in Materials and Methods to detect cytotoxicity of CAK cells against HepG2 cells following pre-treatment of the HepG2 cells without or with gemcitabine (0, 0.01, 0.1 µg/ml). Effector (CAK): target (HepG2) cell ratio was 20:1. All data are expressed as the average percentage specific cytotoxicity \pm SE of triplicate wells and are representative of three independent experiments. * $p < 0.05$, when comparing gemcitabine alone and gemcitabine with CAK.

Therefore, we investigated whether recombinant sMICB inhibits activated lymphocyte- and NK cell-mediated antitumor cytotoxicity. When NKG2D-MICB interactions were blocked with saturating amounts of recombinant sMICB, the killing of gemcitabine-treated HepG2 cells was significantly reduced in a dose-dependent manner (Figure 9). We also investigated whether gemcitabine treatment affects MICB shedding in HepG2 cells. We found that MICB concentrations in culture medium from control HepG2 cells increased over the time course of the assay, and the presence of increasing concentrations of gemcitabine during culture had a negligible effect on MICB concentration (Figure 10), indicating that gemcitabine does not affect MICB shedding from HepG2 cells.

Discussion

In this study, we found that surface protein expression of MICA/B and mRNA of MICB was increased after exposure of HepG2 cells to gemcitabine. We also found that gemcitabine did not increase MICB shedding from HepG2 cells. Up-regulation of the NKG2D ligand, MICB, has been reported to occur following severe stresses such as heat shock, bacterial or viral infection, DNA damage, oxidative stress, and treatment with retinoic acid or histone deacetylase inhibitors (8, 15).

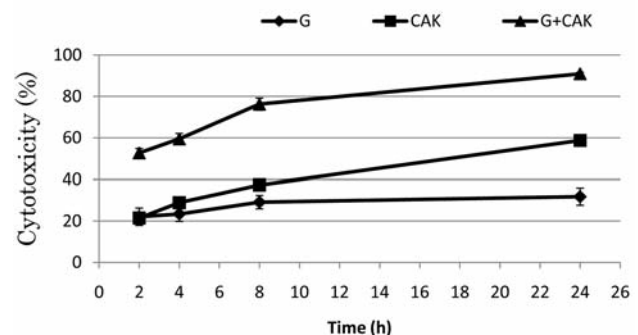


Figure 7. Time course of activated killer cell (CAK) cytotoxicity against HepG2 cells with and without gemcitabine (G). HepG2 cells were incubated in the presence or absence of gemcitabine with or without killer cells for the indicated time. The percentage of cytotoxicity was measured by ATD assay as described in the Materials and Methods.

We have shown that gemcitabine-induced effects on MICA/B expression enhance the susceptibility of HepG2 tumor cells to the cytotoxic actions of CAK cells. We conclude from this result that the interaction between NKG2D and its ligands, MICA and MICB, play a role in CAK-mediated lysis of HepG2 cells, and that the increased susceptibility of gemcitabine-treated cancer cells to CAK

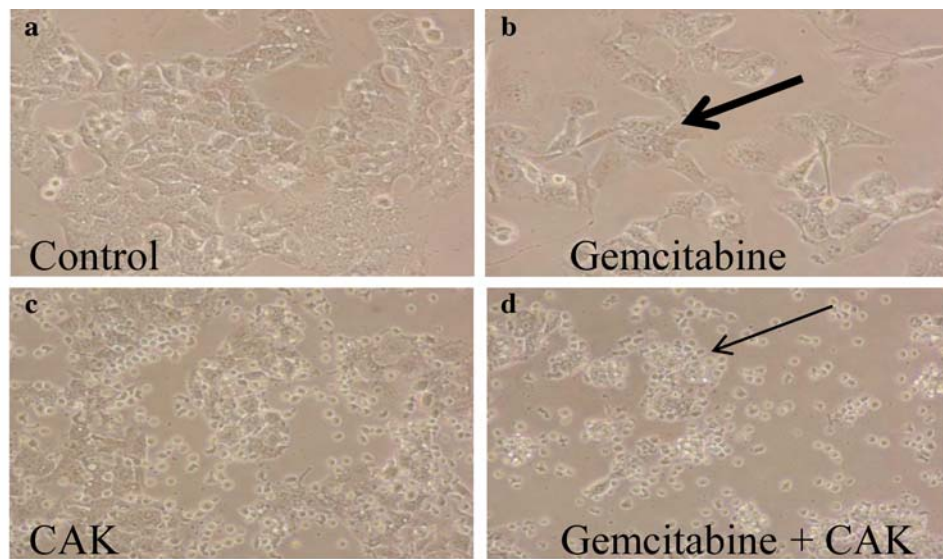


Figure 8. Effect of gemcitabine and CAK cells on HepG2 cell morphology. Morphology of tumor cells treated with medium alone (a), gemcitabine alone (b), CAK cells alone (c), or a combination of gemcitabine and CAK cells (d) for 24 h and morphology observed under an inverted microscope. Images shown are $\times 200$ magnification. Bold arrow: HepG2 cells; thin arrow: CAK cells.

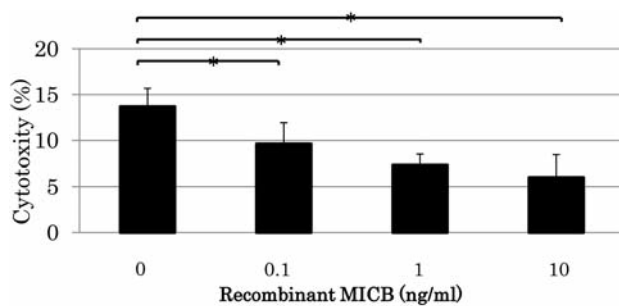


Figure 9. Effect of recombinant MICB on CAK cytotoxicity against HepG2 cells. HepG2 cells were incubated with or without soluble recombinant MICB (sMICB) for 4 h at 37°C before adding CAK cells. Cells were incubated for a further 4 h prior to performing an ATD assays as described in the Materials and Methods. All data are expressed as the average percentage specific cytotoxicity \pm SE of triplicate wells and are representative of three independent experiments. $*p < 0.05$, when comparing control (sMICB=0) and sMICB-treated groups.

cell-induced cytotoxicity may be mediated by up-regulation of NKG2D ligands. Given the extensive distribution of NKG2D in immune cells (e.g. NK, T-, and $\gamma\delta$ T-cells) and the antitumor significance of interactions between NKG2D and its cognate ligands, it is reasonable to predict that gemcitabine may thus augment CAK immunotherapies.

The CAK cells used in this study were a heterogeneous population including activated NK cells and T-cells. The cytotoxicity of both cell populations is at least partly dependent on the NKG2D-MICA/B systems. Importantly,

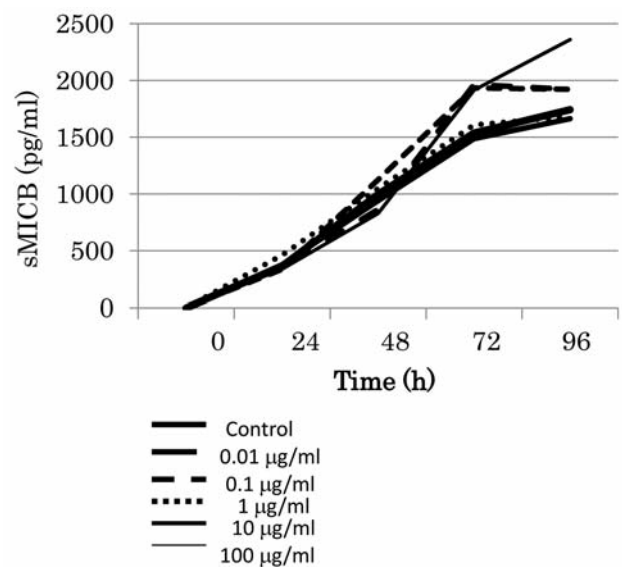


Figure 10. Soluble MICB induction is not affected by gemcitabine. Culture supernatants from HepG2 cells (5×10^4 cells/ml) were treated with or without gemcitabine for the indicated time before being analyzed for sMICB concentration. Data are expressed as the average concentration of sMICB from three independent experiments.

NKG2D can help NK cells to overcome inhibitory signals, and substantially enhances the cytotoxicity of activated T-cells against tumor cells independently of any interaction with TcR class I (16). Activated expanded CD8⁺ T-cells can also lyse tumor cells through NKG2D systems independently

of the TcR class I system (17, 18), demonstrating that NK, $\gamma\delta$ T- and activated expanded CD8⁺ T-cells can all act *via* the NKG2D system and independently of interactions with the TcR HLA class I system.

In conclusion, we have shown that gemcitabine induces MICA/B expression in HepG2 cells, resulting in synergistic enhancement of the cytotoxic effects of CAK cells. Therefore, the combination of gemcitabine with CAK cell immunotherapy may have clinical significance for therapy of hepatocellular carcinoma.

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