

Macrophage Inflammatory Protein Derivative ECI301 Enhances the Alarmin-Associated Abscopal Benefits of Tumor Radiotherapy

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Abstract

Radiotherapy can produce antitumor benefits beyond the local site of irradiation, an immune-based phenomenon known as the abscopal effect, but the mechanisms underlying these benefits are poorly understood. Preclinical studies of ECI301, a mutant derivative of macrophage inhibitory protein-1 α , have shown that its administration can improve the antitumor effects of radiotherapy in a manner associated with a tumor-independent abscopal effect. In this article, we report that i.v. administration of ECI301 after intratumoral injection of tumor cell lysates can inhibit tumor growth, not only at the site of injection but also at nontreated sites. Effects of the tumor lysate were further recapitulated by i.v. administration of the alarmins HSP70 or HMGB1, but not HSP60, and combinations of ECI301 + HSP70 were sufficient to inhibit tumor growth. Although injection of ECI301 + HMGB1 did not inhibit tumor growth, we found that administration of a neutralizing HMGB1 antibody neutralized the cooperative effects of ECI301 on tumor irradiation. Moreover, mice genetically deficient in TLR4, an immune pattern receptor that binds alarmins, including HMGB1 and HSP70, did not exhibit antitumor responses to irradiation with ECI301 administration. Although ECI301 was cleared rapidly from peripheral blood, it was found to bind avidly to HSP70 and HMGB1 *in vitro*. Our results suggest a model in which sequential release of the alarmins HSP70 and HMGB1 from a tumor by irradiation may trap circulating ECI301, thereby licensing or restoring tumor immunosurveillance capabilities of natural killer cells or CD4⁺ and CD8⁺ T cells against tumor cells that may evade irradiation. *Cancer Res*; 74(18); 5070–8. ©2014 AACR.

Introduction

There are various limitations to chemotherapy of solid tumors using classic cytotoxic agents that act intensively on rapidly dividing cells. Delivery of agents to the core of the tumor mass is often difficult and tumor cells with slower growth rates or with quiescent cells due to poor nutrient and/or oxygen supply respond to chemotherapy much more modestly, if at all. Over time, tumor cells acquire resistance to various agents. The most serious problem with this kind of treatment, however, seems to be the myelo- and immunosuppressive effect of such agents. As a result, various leukocytes, which play important roles for host defense and should simultaneously cooperate with the treatment, cannot act on the

remaining tumor cells that have evaded treatment. In fact, dramatic reduction of numbers of various leukocytes are normally observed during and after chemotherapy and, as a result of the extensive treatments, the numbers of lymphocytes become very low and do not recover to normal levels (1). Incidentally, cooperative action of the host defense mechanism is essential for eradication of infected bacteria when treating patients with antibiotics, and, hence, infection control by antibiotics is difficult in compromised hosts even when the patient has a minor defect like opsonin deficiency (2).

On the other hand, there are also limitations in curative radiotherapy, because in this case, also, slowly dividing cells or cells with low metabolic activity are less sensitive to ionizing radiation. In addition, tumor cells under hypoxic conditions are insensitive to irradiation that mediates most effects through active oxygen species such as hydroxyl radicals generated from water and oxygen by radiolysis. Tumor cells express hypoxia inducible factor-1 α that allows the cells to adapt to low-oxygen conditions (3, 4). One notable advantage of local radiotherapy, in contrast with systemic chemotherapy is, however, that the host defense system can act on the remaining cells soon after irradiation. In fact, inflammation induced by irradiation is known to play a significant role in remission of tumor growth and to affect the prognosis of the patient.

Various alarmins or danger-associated, molecular-pattern molecules (DAMP) such as heat shock proteins (HSP),

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high-mobility group box-1 protein (HMGB1), S100 proteins, and hepatoma-derived growth factor are reportedly released by irradiation (5–8). HSP70 is a molecular chaperone that translocates to the outer leaflet of the plasma membrane during tumor cell death and is released from the necrotic cells (9, 10). This protein works to activate natural killer (NK) cells and to present associated peptides to antigen-presenting cells (APC; ref. 11). HMGB1, a 215 amino acid chromatin-binding protein overexpressed in tumor cells, is released from dying cells and acts at several levels of the immune response, including promotion of inflammation by recruitment and activation of dendritic cells and tumor-specific T cells (12–14). However, a series of these events after irradiation is usually not enough to eradicate the remaining tumor cells.

Previously, we showed that ECI301, a recombinant variant of human MIP-1 α (CCL3), enhanced the antitumor radiation efficacy of subcutaneously growing tumor cells (15). In nearly half of mice treated by this combination therapy, tumors were completely eradicated and rechallenged syngenic tumor cells were rejected. In similar experiments, reduction of tumor growth distant from the irradiated site was found to be consistently induced and this phenomenon (called the abscopal effect, an effect distal to the target), observed in BALB/c and C57BL/6 mice, was tumor-type independent. The effect of ECI301 was shown to be diminished by an antitumor chemotherapeutic agent, docetaxel, when administered before ECI301 injection, suggesting that the agent affected the recruitment and function of leukocytes, which are essential for tumor eradication.

Because enhancement of tumor growth inhibition by ECI301 was not attributable to radiation alone but also shown to other factors such as the heat generated from high-frequency alternating current, namely radiofrequency ablation (RFA; ref. 16), we supposed that substances released during treatment may cause inflammation at tumor-bearing sites and play important roles in tumor growth inhibition. In this article, we show that intratumor injection of tumor lysate mimics these treatments, and HSP70 and HMGB1, which seem to be induced by local antitumor treatments, play crucial roles in tumor regression and reduction of tumor growth at nontreated sites (i.e., by the abscopal effect that does not depend on radiation), probably by binding administered ECI301. These results reveal an underlying mechanism of the combination therapy of radiation or RFA treatment and i.v. injection of ECI301.

Materials and Methods

Materials

Recombinant ECI301, a 69 amino acid variant of human MIP-1 α carrying a single amino acid substitution of Asp27 of MIP-1 α to Ara, was generated using an *S. pombe* expression system (Asahi Glass Co. Ltd.) and purified to homogeneity (15). Recombinant hMIP-1 α was obtained using a *Brevibacillus choshinensis* expression system (Protein Express) or purchased from PeproTech. Migration rate of CCR1-expressing Jurkat cells toward ECI301 was slightly higher than that toward MIP-1 α at lower concentrations when assayed using TAXIScan (17). Bovine thymus HMGB1 was obtained from ShinoTest, and

recombinant mouse HSP60, HSP70-A1, and HSP90 (all low endotoxin) were from Enzo Biochem. The following antibodies were used unless otherwise stated: Chikin anti-HMGB1 polyclonal antibody (IgY fraction), mouse monoclonal anti-hMIP-1 α (R&D Systems), anti-HSP70 antibodies (StressMarq) and biotin-labeled, rabbit polyclonal anti-MIP-1 α antibody (R&D Systems).

Mice

Six-week-old male BALB/c, female C57BL6, male C3H/HeN, and male C3H/HeJ mice were purchased from Nippon SLC and housed in a barrier system with controlled light (12L:12D) and temperature (22°C \pm 2°C). They were fed a diet of mouse chow and water *ad libitum* and used at 7-weeks-old. All animal experiments were carried out in accordance with the guidelines for animal experiments at the University of Tokyo (Bunkyo, Tokyo, Japan).

Tumor cells

As described previously (15), Colon26 adenocarcinoma cells were provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan) and MethA fibrosarcoma and Lewis lung carcinoma (LLC) cells were obtained from the American Type Culture Collection. FM3A mouse mammary carcinoma cells were obtained from the Human Science Research Resource Bank (Osaka, Japan), which is resistant to 4-carbamoylimidazolium 5-olate. The cultures were maintained in RPMI-1640 medium supplemented with 10% FBS, for no longer than 12 weeks after recovery from frozen stocks. Culture conditions were 37°C under humidified 5% CO₂.

Tumor cell growth *in vivo*

Before tumor implantation, cells were trypsinized and filtered through a 70- μ m pore size cell strainer. When the mass of solid tumor at the right flank reached 5- to 10-mm diameter (14–18 days after implantation), mice with similar-sized tumors were selected and used for the experiments.

The tumor volume was calculated using the following formula:

$$(\text{Tumor volume}) = (\text{Major axis}) \times (\text{Minor axis})^2 \times 0.5236.$$

Tumor irradiation

Mice bearing tumors were held in the decubitus position, and ionizing radiation (6 MeV-electron beam) was delivered to the right flank from just above the tumor (15).

In vitro binding study of ECI301 to HSP70 by immunoprecipitation analysis

Ten milligrams magnetic beads in the Co-IP Kit (Invitrogen) and 50 μ g of mouse monoclonal anti-hMIP-1 α or anti-HSP70 antibody were conjugated according to the instruction manual and the beads were suspended in 1-mL buffer. Fifty microliters of antibody coupled beads (1 mg/5 μ g antibody), and 25 μ L each of ECI301 (600 ng), HSP70-A1 (6 μ g), biotin-labeled, rabbit polyclonal anti-MIP-1 α antibody (6 μ g), hMIP-1 α (600 ng) all in PBS with 0.01% polysorbate80 or their combinations were mixed (total volume, 100 μ L) and incubated at 4°C in a rotator

for 24 hours. The beads were washed carefully and subjected to SDS-PAGE at 200V for 35 minutes after incubation in reducing buffer at 70°C for 10 minutes. Immunoblotting was performed using iBlot (Invitrogen) on polyvinylidene difluoride (PVDF) membranes according to the instruction manual. After blocking with ChemiBlocker, the membrane was stained at room temperature for 60 minutes with biotin-labeled, rabbit polyclonal anti-MIP-1 α antibody and then under the same conditions with horseradish peroxidase (HRP)-labeled streptavidin (Jackson; first staining). The PVDF membrane was further stained using mouse anti-HSP70 antibody and HRP-labeled anti-murine immunoglobulin G (American Quaiex; second staining). Chemiluminescence excited with the ECL Western Blotting Detection system (GE Healthcare) was detected using image analyzer LAS4000 Mini (Fujifilm).

Binding analysis of ECI301 to HMGB1 using Biacore

Changes in surface plasmon resonance were measured using Biacore X (GE Healthcare), in which immobilized ECI301 or MIP-1 α (Peprotech) was used as a "ligand" on the sensor chip CM5 (using the Thio Coupling Kit) and a series of concentration of HMGB1 was used as "analytes." Refraction change at the surface was detected and recorded as resonance units (RU) in the control software supplied and evaluated by fitting algorithms. RUs were measured during the binding and washing periods, and the binding kinetics were analyzed using BIAevaluation Software, version 3.0. RU values were used to determine dissociation constant (Kd).

Statistical analysis

Unless otherwise stated, data are presented as mean \pm SE. For comparisons between groups in the *in vivo* study, we used ANOVA. The significance of the difference between the means of two variables was determined by the Dunnett test unless otherwise stated. A *P* value of <0.05 was considered significant.

Results

Effect of i.v. administered ECI301 on tumor growth after intratumor injection of tumor lysate

We predicted the presence of proteins that works in synergy with ECI301 to promote inflammation at the local antitumor-treated site. This prediction was made based on previous findings, such as ECI301-enhanced efficacy of antitumor radiation with the abscopal effect (15) and on the reported evidence of augmentation by ECI301 of RFA-induced antitumor effect against non-RFA-treated hepatocellular carcinoma cells (16). We examined whether intratumor injected tumor lysate could recapitulate the effect of radiation or RFA in the combination treatment. Colon26 adenocarcinoma cells were implanted subcutaneously in the right and left flanks of BALB/c mice and tumors were allowed to grow until the tumor size of the right flank reached 5- to 10-mm diameter. Tumor lysate obtained from Colon26 cells by sonication was injected to the center of the tumor mass in the right flank. Animals received ECI301 intravenously once a day for 5 consecutive days starting from immediately after administration of tumor lysate. As shown in Fig. 1, the rate of tumor growth was

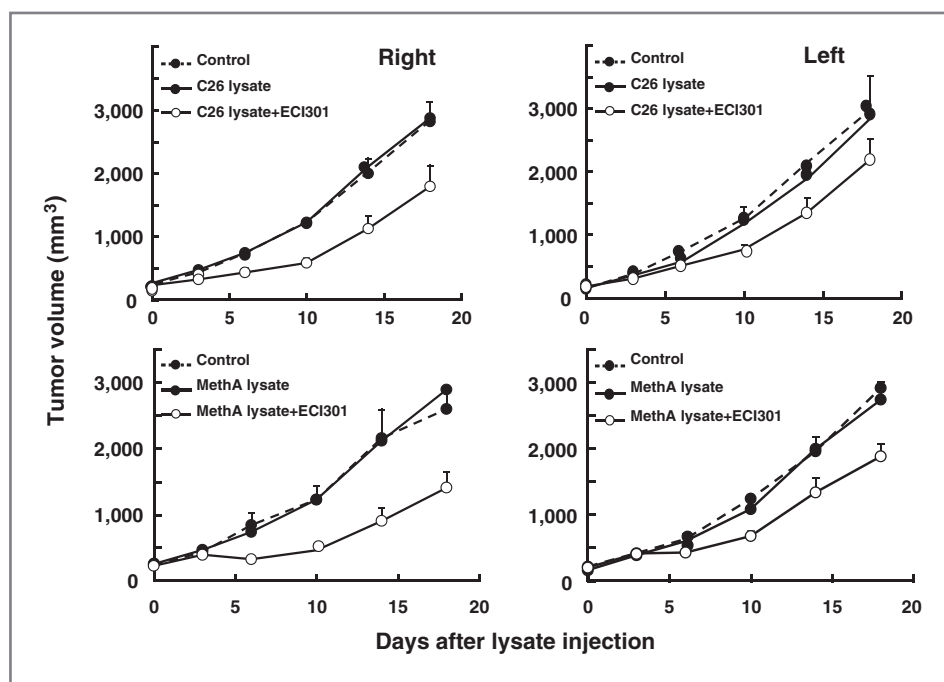
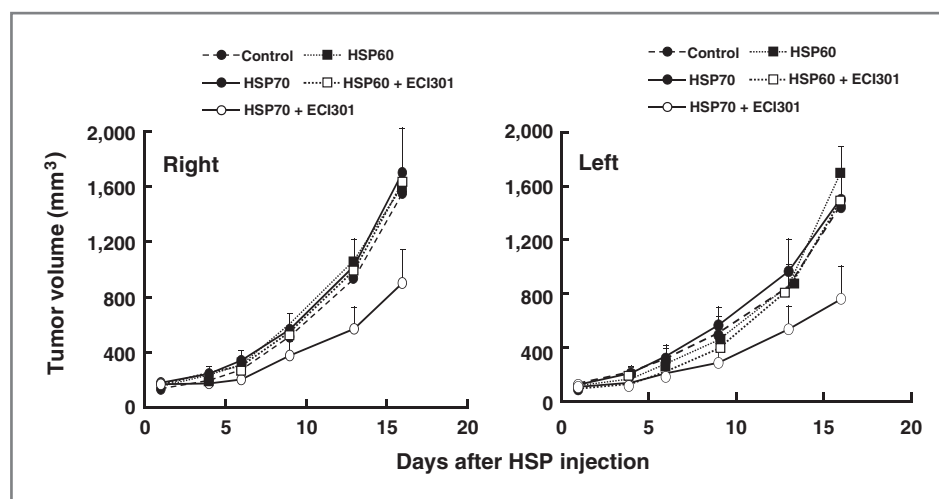


Figure 1. Tumor growth after intratumor injection of tumor lysate followed by i.v. administration of ECI301. Figures show time course of Colon26 growth at tumor lysate injected (right) or noninjected sites (left). Colon26 cells were implanted subcutaneously in the right (4×10^5 cell/mouse) and left flanks of BALB/c mice (2×10^5 cell/mouse). When the tumor size of the right flank reached 5- to 10-mm diameter (14 days after implantation), tumor lysate (●) from Colon26 (top figures) or MethA cells (bottom figures) obtained by sonication (50 μ L, from 10^6 cells/mL PBS) was injected to the center of the tumor mass in the right flank (day 0). Mice received ECI301 (2 μ g/0.2 mL PBS/mouse) intravenously once a day for 5 consecutive days starting from immediately after administration of tumor lysate (○). Tumor growth without any treatment is shown as control (●). Points and bars, mean tumor volume and SE ($n = 7$).

Figure 2. Effect of i.v. administered ECI301 on tumor growth after intratumor injection of HSPs. Figures show time course of Colon26 growth at HSP injected (right) or noninjected sites (left). Either HSP60 (■) or HSP70 (●; 50 ng/50 μ L/mouse) was injected to the center of the tumor mass in the right flank (day 0). Mice received ECI301 (2 μ g/mouse) intravenously once a day for 5 consecutive days starting from immediately after administration of HSP (HSP60+ECI301, □; and HSP70+ ECI301, ○). Tumor growth without any treatment is shown as control (●). Points and bars, mean tumor volume and SE ($n = 7$).



significantly inhibited not only at the injected site (top right) but also at the noninjected site (top left). Administration of ECI301 without the tumor lysate injection caused no noticeable effect on tumor growth (15). The effect of tumor lysate was found not to be restricted to that of Colon26, and other tumor cell lysates such as from MethA fibrosarcoma cells were also effective (Fig. 1, bottom left and right).

Effective replacement of tumor lysate with HSP70

It has been proposed that immunogenic tumor cell death is characterized by a temporal sequence of events, including early translocation and exposure of HSPs in the cell surface and their release from the cells, followed by the late release of HMGB1 from dying cells (9, 10, 18). Among alarmins that might be generated in the tumor-bearing site, we first focused on HSPs and carried out similar experiments as in Fig. 1 using HSPs instead of tumor lysate. Intratumor injection of HSP70 followed by 5-dose i.v. administration of ECI301 (once a day for 5 consecutive days), resulted in significant tumor growth inhibition not only at the injected site (Fig. 2, right) but also at the nontreated site (Fig. 2, left). HSP70 without ECI301 administration and HSP60 with or without ECI301 administration (Fig. 2) did not show such an effect. In other experiments, when Colon26 cells were implanted in the right flank of BALB/c mice alone, the tumor in 1 of 5 mice was completely eradicated by this treatment and the mouse survived beyond 60 days, significantly outliving all other mice.

We further found that i.v. injected mixtures of HSP70 and ECI301 (once a day for 3 consecutive days) inhibited Colon26 growth without pretreatments such as radiation, RFA, or intratumor injection of tumor lysate or HSP70 (Fig. 3 top). A statistically significant antitumor effect was found when a mixture of ECI301 and HSP70, was given at 2 and 0.5 μ g per mouse ($P < 0.01$), or 10 and 2.5 μ g per mouse ($P < 0.05$), among various tested ratios and doses (ECI301/HSP70: 2/0.125, 2/0.5 and 2/2.5, or 0.4/0.1, 2/0.5, and 10/2.5 μ g/mouse). The effect was restricted to HSP70 among the HSPs tested and inhibition was not observed by i.v. administration of a series of mixtures of ECI301/HSP60 (2/0.1, 2/0.5, and 2/2.5 μ g/

mouse), or that of ECI301/HSP90 (2/0.5 μ g/mouse; Supplementary Fig. S1A and S1B). It is interesting to note that MIP-1 α could not mimic the effects of ECI301, and i.v. injection of a mixture of ECI301/MIP-1 α (0.4/0.1 or 2/0.5 μ g/mouse) did not

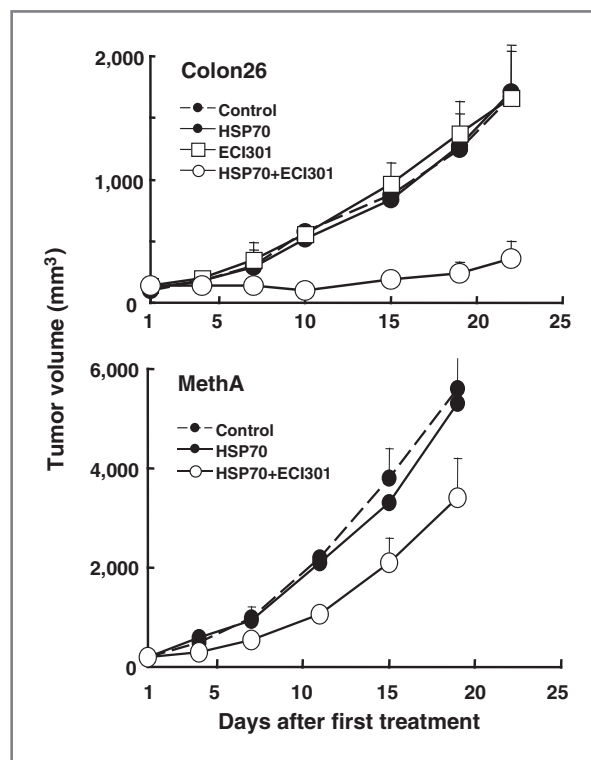


Figure 3. Effect of i.v. administered mixture of ECI301 and HSP70 on tumor growth. Colon26 cells (top, 4×10^5 cell/mouse) or MethA cells (bottom, 1×10^5 cell/mouse) were implanted in the right flank of BALB/c mice. When the tumor size reached 5- to 8-mm diameter (14 days), animals received 0.5- μ g HSP70 (●), 2- μ g ECI301 (□) or a mixture of 0.5- μ g HSP70 and 2- μ g ECI301 in 0.2 mL PBS, intravenously three times at days 0, 1, and 2 (HSP70+ECI301, ○). Tumor growth without any treatment is shown as control (●). Points and bars, mean tumor volume and SE ($n = 6$). Experiments with HSP70 were independently repeated five times and representative results are shown.

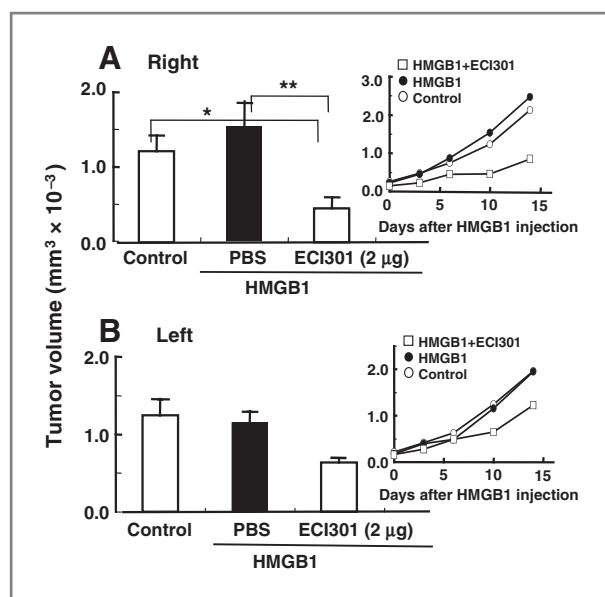


Figure 4. Effect of i.v. administered ECI301 on tumor growth after intratumor injection of HMGB1. Mean tumor volume \pm SE of 7 mice at 10 days after intratumor injection of HMGB1 and time course of Colon26 growth (inset) are shown. Colon26 cells were implanted in the right (2×10^5 cells; A) or the left flanks (1×10^5 cells; B) of BALB/c mice. When the tumor size of the right flank reached approximately 10-mm diameter (18 days), HMGB1 (50 ng/50 μ L/mouse) was injected to the center of tumors at the right flank (\bullet), followed by i.v. administration of 2 μ g per mouse ECI301 (\square) once a day for 5 consecutive days, starting 3 hours after HMGB1 injection. Tumor growth without treatment is shown as control (\circ). Significant differences between each group are indicated by **, $P < 0.01$ or *, $P < 0.05$ (Dunnett). Experiments were independently repeated and representative results are shown.

influence Colon26 growth in BALB/c mice (Supplementary Fig. S1C). The tumor growth inhibitory effect of the mixture of ECI301 and HSP70 was also found with MethA-bearing BALB/c mice (Fig. 3, lower).

Effect of intratumor injection of HMGB1 followed by i.v. administered ECI301 on tumor growth

We detected a 30 kDa band of HMGB1 in the sonicated tumor lysate from Colon26 cells by Western blottings: both in the supernatant and the precipitate obtained after centrifugation at $20,000 \times g$ for 15 minutes (data not shown). We, therefore, examined whether HMGB1 could also replace tumor lysate or HSP70. Similar experiments using a Colon26 cells/BALB/c mice system as described above were performed using HMGB1 instead of HSP70. When tumor size of the right flank reached approximately 10-mm diameter, HMGB1 (50 ng/mouse) was given to the center of the tumor mass in the right flank. Animals received ECI301 intravenously five times (once a day for 5 consecutive days) after HMGB1 administration. As shown in Fig. 4, tumor growth in both flanks was markedly inhibited by the combination treatment of HMGB1 injected to the right-side tumor and i.v. administered ECI301. HMGB1 injected to the tumor mass without i.v. administration of ECI301 did not influence tumor growth (Fig. 4, PBS).

Effect of anti-HMGB1 antibody on enhancement of tumor growth inhibition by the combination treatment of radiation and ECI301

To confirm the role of HMGB1 in the combination treatment of ECI301 administration after local irradiation, we investigated the effect of anti-HMGB1 antibody on enhanced tumor growth inhibition. In these experiments, mice received anti-HMGB1 antibody by i.p. injection (100 μ g/mouse) two or three times either; 3 hours before irradiation along with the first ECI301 administrations; or in addition 3 hours before the fifth ECI301 administration. In the right-flank tumor in which radiation was delivered (Fig. 5A), anti-HMGB1 antibody (administered three times) diminished the ECI301 effect and radiation effect, and tumor growth became comparable with that without treatment (control). In the left-flank tumor also (Fig. 5B), significant differences were ablated by the anti-HMGB1 antibody. Similar results were also obtained without a third injection of the antibody.

Effect of ECI301 with local irradiation on tumor growth in functionally defective TLR4 mice

Because it is known that the Toll-like receptor 4 (TLR4) reacts with HSP70 or HMGB1 (19–21), which in turn stimulates

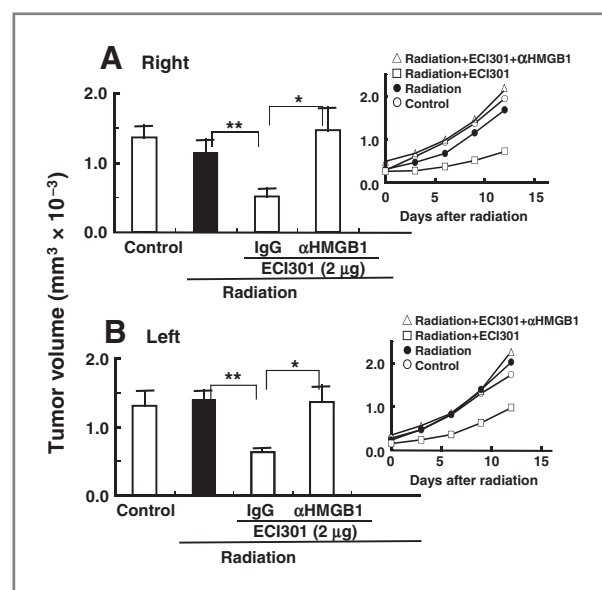


Figure 5. Effect of anti-HMGB1 antibody on the combination treatment of radiation and ECI301 administration. Mean tumor volume \pm SE of 7 mice at 9 days after irradiation and time course of Colon26 growth (inset) are shown. Colon26 cells were implanted in the right (2×10^5 cell; A) and left flanks of BALB/c mice (1×10^5 cell; B). Animals received a single dose of 6 Gy radiation at the right flank 18 days after implantation (\bullet), followed by i.v. administration of 2 μ g per mouse ECI301 (\square) once a day for 5 consecutive days, starting 20 hours after irradiation. Anti-HMGB1 antibody (100 μ g/mouse) was injected into the peritoneal cavity three times; 3 hours before irradiation; before the first and the fifth ECI301 administrations (Δ). Tumor growth without treatment is shown as control (\circ). Significant differences from the irradiated group or from irradiation/ECI301/antibody group are indicated by **, $P < 0.01$ or *, $P < 0.05$ (Dunnett). Similar results were obtained with the independently repeated experiment and also without a third injection of the antibody.

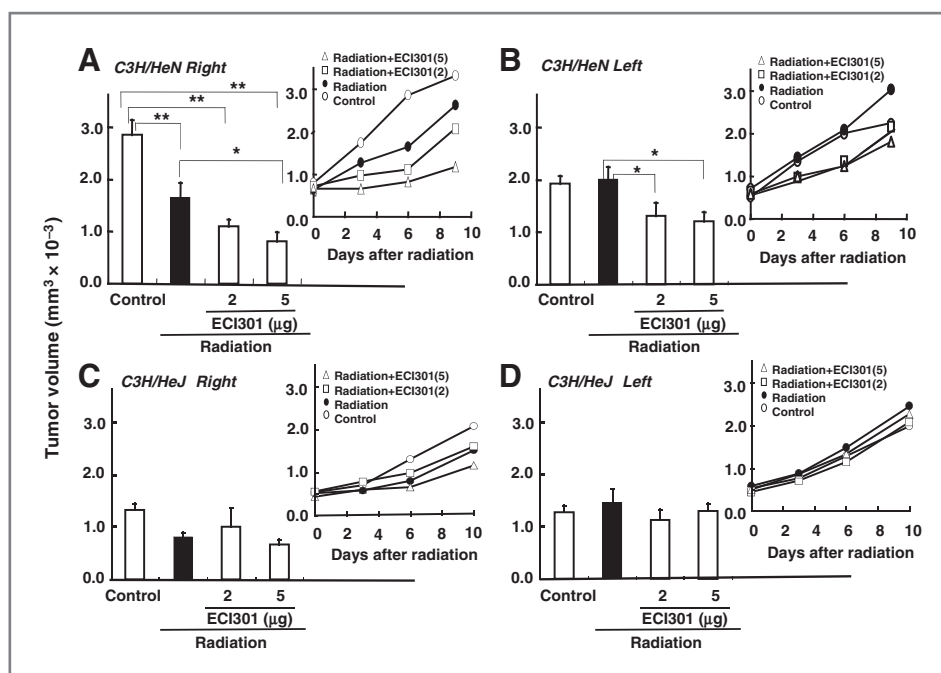


Figure 6. Tumor growth in C3H/HeN and C3H/HeJ mice treated with a combination of radiation and ECI301 administration. The mean tumor volume at 6 days after irradiation in C3H/HeN (A and B) or C3H/HeJ mice (C and D) and the time course of tumor growth in C3H/HeN mice or C3H/HeJ mice (insets) are shown. Each column, mean tumor volume \pm SE ($n = 7$). FM3A carcinoma cells were implanted subcutaneously in the right (A; 8×10^6) and the left flanks (B; 4×10^6) of C3H/HeN and in the right (C; 8×10^6), and the left flanks (D; 4×10^6) of C3H/HeJ mice. A single dose of 6 MeV electron beam (animal received 6 Gy) was delivered to the tumor 15 days after tumor implantation (●), followed by i.v. administration of 2 μ g (□) or 5 μ g per mouse ECI301 (Δ), once a day for 5 consecutive days. Tumor growth without treatment is shown as control (○). The units of numbers in parentheses (inset) are μ g. Significant differences between each group are indicated by **, $P < 0.01$ or *, $P < 0.05$ (Dunnett). Experiments were independently repeated three times and representative results are shown.

dendritic cells and other immune cells, we studied whether the combination therapy with radiation and ECI301 is effective in the mice whose TLR4 function is defective. In these experiments, FM3A carcinoma cells were implanted subcutaneously in both flanks of C3H/HeN and C3H/HeJ mice. Fifteen days after implantation when the tumor size reached approximately 10-mm diameter, mice received 6 Gy at the right-flank tumor and ECI301 was administered i.v. once a day for 5 consecutive days starting 20 hours after irradiation. In control C3H/HeN mice, enhanced antitumor radiation efficacy and induction of the abscopal effect were observed with i.v. administered ECI301 (Fig. 6A and B). In contrast, neither significant enhancement of antitumor radiation efficacy nor induction of the abscopal effect with ECI301 was observed in C3H/HeJ (Fig. 6C and D).

Tumor-specific IFN γ production by splenocytes from tumor-bearing mice after combination treatment

In a previous study (15), we showed by depletion of CD4⁺ T cells, CD8⁺ T cells, and NK1.1 cells that these cells play important roles in tumor regression during the combination treatment of radiation and ECI301 administration. To further confirm the involvement of cytotoxic T cells, we evaluated, using ELISPOT assay, whether treatment with ECI301 following ionic irradiation increases the number of cells that can promote tumor-specific IFN γ production in the spleen, by

using LLC cell-bearing C57/BL6 mice. We found that splenocytes from tumor-bearing mice 3 days after ECI301 administration (4 days after irradiation) contained significantly higher numbers of cells that can secrete IFN γ (Supplementary Fig. S2). This response was specific to a particular tumor-bearing type (LLC), and was not detected spontaneously or in response to another heterotypic tumor cell type (MethA). Administration of ECI301 without irradiation did not increase the number of cells that capable of secreting tumor-specific IFN γ in the spleen.

Binding of ECI301 to HSP70 and HMGB1 *in vitro*

It was reported that highly purified HSP70 and HMGB1 can stimulate the immune system only by binding other molecules (22–24). We tested whether ECI301 binds to these molecules. To find interactions between ECI301 and HSP70, we performed coimmunoprecipitation analysis using Dynabeads coupled with anti-MIP-1 α antibody or anti-HSP70 antibody (anti-MIP-1 beads or anti-HSP beads, respectively). A series of mixtures containing anti-MIP-1 beads together with either ECI301 alone, ECI301+HSP70, ECI301+anti-MIP-1 α antibody, and a mixture containing anti-HSP beads together with either HSP70 alone, HSP70+ECI301, or HSP70+hMIP-1 α , were incubated at 4°C for 24 hours in a rotator. As shown in Fig. 7, lane 2, HSP70 was precipitated with anti-MIP-1 beads in the presence of ECI301. On the

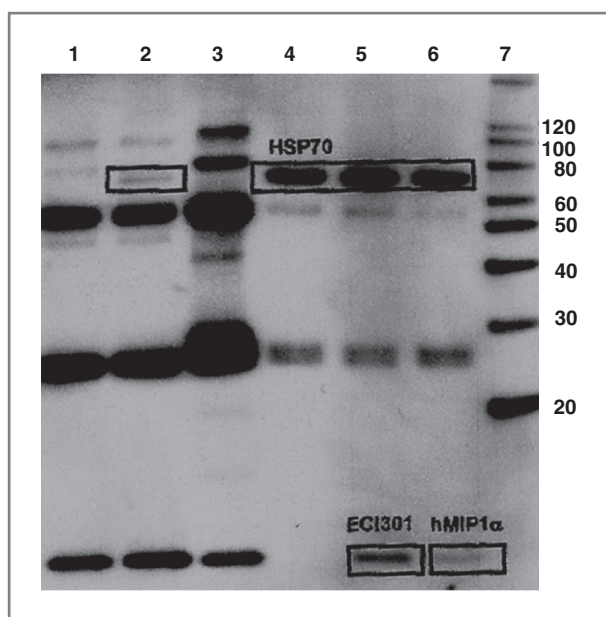


Figure 7. Coprecipitation of ECI301 and HSP70 or HMGB1. The anti-hMIP-1 α antibody (murine)-coupled magnetic beads were used for lanes 1–3 and incubated with ECI301 alone (lane 1), ECI301+HSP70 (lane 2), and ECI301+biotin-labeled anti-MIP-1 α antibody (lane 3). The anti-HSP70 antibody (murine)-coupled magnetic beads were used for lanes 4–6 and incubated with HSP70 alone (lane 4), HSP70+ECI301 (lane 5), HSP70+hMIP-1 α (lane 6). Molecular markers were shown in lane 7 (kDa). The bottom bands in lanes 1–3, and 5 are those of ECI301 and in lane 6, is that of MIP-1 α , all of which were detected by the first staining. The bottom bands of ECI301 and MIP-1 α in lanes 5 and 6, respectively (boxed), were those coprecipitated with HSP70. In lanes 2 and 4–6, the upper boxed bands are of HSP70, which appeared from the second staining. The HSP70 band in lane 2 is the one coprecipitated with ECI301. The upper two major bands seen in lane 1 and corresponding bands in lanes 2–6 are those of the heavy and light chain of antibodies (anti-MIP-1 α antibody from beads/lanes 1 and 2; anti-MIP-1 α antibody from beads + biotin-labeled anti-MIP-1 α antibody added to the sample/lane 3; anti-HSP70 antibody from beads/lanes 4–5). These bands were not detected by the first staining except those in lane 3.

other hand, ECI301 was coprecipitated with HSP70 bound to anti-HSP beads (lane 5). When MIP-1 α was used instead of ECI301, it also precipitated with anti-HSP beads but to a lower extent (lane 6). These results indicate that HSP70 can form a stable complex with ECI301 and, to a lesser extent, with MIP-1 α .

We could not show any interaction between ECI301 and HMGB1 *in vitro*, by similar coprecipitation studies using anti-MIP-1 beads. We then evaluated changes of surface plasmon resonance upon interaction between these molecules using Biacore X (GE Healthcare). As a "ligand" on the sensor chip, ECI301 or MIP-1 α was immobilized and responses to a series of concentrations of HMGB1 were determined. The K_d value of HMGB1 obtained with ECI301 was 3.7×10^{-8} . The value obtained with MIP-1 α (3.0×10^{-7}) was 10-fold higher than that obtained with ECI301. The results indicate that ECI301 binds also to HMGB1. This binding affinity and stability were much stronger than those of MIP-1 α .

Discussion

In this study, we have presented evidence to support the notion that HSP70 and HMGB1, alarmins known to be released early and lately from dying tumor cells after irradiation or other local anticancer treatments, bind to i.v. administered ECI301 and play a crucial role in tumor regression by stimulating host defense mechanisms. As we reported previously (15), a significant number of CD4⁺ and CD8⁺ T cells infiltrated tumors at the irradiated and nonirradiated sites when ECI301 was administered after irradiation, and that depletion of any of CD8⁺T, CD4⁺T, or NK1.1 cells using antibodies diminished the enhanced antitumor and abscopal effects by ECI301. Involvement of T cells in the enhancement of radiation efficacy at the irradiated site was further confirmed by rejection of rechallenged syngenic tumor cells in the tumor-eradicated mice. We showed in this article that splenocytes in tumor-bearing mice treated with radiation and ECI301, contained a significant number of cells with potential to secrete IFN γ upon exposure to the tumor cells, suggesting that systemic tumor-specific immunity is inducible by such CD8⁺ T cells infiltrating to the tumor-bearing site.

A chaperone protein, HSP70 is reported to be overexpressed in many tumors (25) especially at the plasma membrane (26). Overexpression of HMGB1 has also been reported in most tumor cells (27, 28), and has been shown to be closely associated with various characteristics of tumor cells, including unlimited cell division (29). This may explain why ionizing radiation delivered to a tumor-bearing site but not to normal tissues is required to elicit the effect of ECI301 (15). We found that HMGB1 was present in tumors (in lysates) both in free and bound forms, though an increase of serum levels of HMGB1 after radiation treatment or following ECI301 administration was not detected (unpublished observation). The result suggests that the enhancement by ECI301 only takes place inside, or close to, the tumor-bearing site at least in the case of HMGB1. It is well known that HMGB1 develops enhanced proinflammatory activity by binding to various cytokines (30) and that necrotic cells deficient for HMGB1 have a strongly reduced ability to activate APCs (31). Although further studies are required to elucidate the relationship between the HSP70/ECI301 and HMGB/ECI301 complexes, the latter complex may work subsequently to the former because HSP70 is released before HMGB1 (9), HSP70 induces HMGB1 (18), and anti-HMGB1 antibody diminishes the effect of combination treatment of irradiation and ECI301.

We found not only that intratumor injection of HSP70 followed by ECI301 administration inhibited tumor growth both in treated and untreated sites, but also that i.v. injected mixtures of HSP70 and ECI301 inhibited tumor growth. A mixture of 2 and 0.5 μ g per mouse ECI301 and HSP70, respectively, was most effective among the various tested ratios and doses. In contrast with HSP70, i.v. injected HMGB1 even together with ECI301 did not inhibit tumor growth, although intratumor injection of this molecule followed by i.v. administration of ECI301 inhibited tumor growth. Although we could not demonstrate stable complex formation between ECI301 and HMGB1 by coimmunoprecipitation studies,

significant interaction between these molecules was shown by changes of surface plasmon resonance upon interaction. The obtained Kd value of HMGB1 with ECI301 was 3.7×10^{-8} , much lower than the value reported for peptide antigen versus anti-peptide (IgG) antibody (between 10^{-5} and 10^{-6}). These results also support the assumption that the enhancement by ECI301 only takes place inside, or close to, the tumor-bearing site in case of HMGB1. Various receptors are known to interact with HSP70 and HMGB1 and, among them, TLR4 and TLR2 seems to be important in mediating HSP70- and HMGB1-induced inflammatory responses (19, 20, 32–37). These receptors are known to respond to HSP70 and HMGB1 only after binding to other molecules (22–24). A HSP70/or HMGB1/ECI301 complex seems to react with TLR4 because, in the TLR4-deficient mice, the combination effect of radiation and ECI301 was not observed. Patients with breast cancer carrying a TLR4 loss-of-function allele are reported to relapse more quickly after radio- and chemotherapy (32).

It is interesting to note that MIP-1 α could not mimic its variant ECI301, and i.v. injected mixtures of HSP70 and MIP-1 α did not inhibit tumor growth. As compared with ECI301, MIP-1 α does not efficiently bind to HSP70 and the Kd value of HMGB1 with MIP-1 α was 10 times higher than ECI301. These results suggest that these lower binding activities make biologic activity of MIP-1 α weaker than ECI301. In fact, we found that MIP-1 α could not reproduce the effect of ECI301 in the combination treatment with radiation (unpublished observation). It has been reported that the basic unit of one MIP-1 α molecule is a dimer, whose primary contact is constituted by two antiparallel β strands formed by an N-terminal loop (38, 39). In MIP-1 α molecules, the hydrogen bonds between Asp27 and Arg46/Arg48 residues, and between Asp6 and Ser33, and the hydrophobic interaction between Tyr28 and Phe24 residues are proposed to contribute to the interaction between the two dimers, and subsequent polymer formation. In ECI301, whose basic unit is also a dimer, Asp27 of MIP-1 α is substituted with Ala. MIP-1 α forms a double-helical polymer burring its receptor-binding sites, whereas substitution of Asp27 with Ala results in irregular polymers with possible dissociation (39). The ECI301 molecule used in the present investigation was found to be a mixture of tetramer (76%), dimer (20%), and polymers larger than tetramer (the remainder) at mg/mL concentration and no significant difference of this ratio was found at 10 μ g/mL (unpublished results). Further study is required to clarify the differences in anticancer activity between ECI301 and MIP-1 α in the combination treatments.

Finally, the difficulty in eradication of tumor cell mass merely by antigen-specific killer T cells or by the combination of alarmins and ECI301 described here must be remarked upon, because even 1 g of tumor mass contains approximately 10^9 cells. For example, the number of mice cured by treatment with both HSP70 and ECI301 was much lower than by the combination of irradiation and ECI301, in which nearly half of the mice were cured (15). Before or during the period when the host-defense system plays its part, other means are essential to eliminate or injure the

bulk of tumor cells, as in the case of antibiotics in bacterial infection, which contribute to eliminate the majority of infected bacteria. The proposed concept, namely, local anticancer treatments that induce inflammation at the tumor-bearing site by irradiation or RFA followed by i.v. administration of ECI301, is based on this assumption, and supported by experimental evidence shown in this and previous articles (15, 16). RFA treatment of tumors is known to induce tumor-specific immune responses and is used as an eradication treatment, though the treatment is often not sufficient to prevent tumor recurrence. ECI301 injection augmented the RFA-induced antitumor effect against non-RFA-treated tumors in the same animal. In contrast with the anti-solid tumor strategies described here, inflammation induced by cyclooxygenase (COX)-2, overexpressed in tumor cells, seems to play a substantial role in "initiation" of oncogenesis, and COX-2 inhibitors are effective in preventing tumor growth by inhibiting proliferation and inducing apoptosis of tumor cells, and release from immunosuppression via regulatory T-cell activation (40). Thus, both strategies do not seem incompatible.

All these studies mentioned above lead to the development of therapeutic applications of ECI301, and a clinical trial using combination of radiation and i.v. administered ECI301 for patients with advanced or metastatic cancer, as well as a clinical trial for RFA followed by i.v. administration of ECI301 for patients with hepatocellular carcinoma are about to commence in the United States and Japan.

Disclosure of Potential Conflicts of Interest

K. Matsushima is a consultant/advisory board member for Kyowa-Hakko Kirin, IDAC Theranostics, and Rohto Pharmaceutical. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Shiraishi, K. Nakagawa, T. Tsuchiya
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Kanegasaki, T. Tsuchiya
Writing, review, and/or revision of the manuscript: S. Kanegasaki, K. Matsushima, T. Tsuchiya
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Kanegasaki, T. Tsuchiya
Study supervision: S. Kanegasaki

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