Enhancement of Antitumor Radiation Efficacy and Consistent Induction of the Abscopal Effect in Mice by ECI301, an Active Variant of Macrophage Inflammatory Protein-1α

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Abstract

Purpose: We studied whether i.v. administration of a chemokine after local tumor site irradiation could prevent remaining, as well as distant, nonirradiated tumor cell growth by leukocyte recruitment.

Experimental Design: Tumors were implanted s.c. in the right or both flanks. After local irradiation at the right flank, ECI301, a human macrophage inflammatory protein-1α variant was injected i.v. Tumor volumes were measured every 3 days after treatment.

Results: In Colon26 adenocarcinoma-bearing BALB/c mice, repeated daily administration (over 3-5 consecutive days) of 2 μg per mouse ECI301 after local irradiation of 6 Gy prolonged survival without significant toxicity, and in about half of the treated mice, the tumor was completely eradicated. Three weekly administrations of ECI301 after local irradiation also led to significant, although less effective, antitumor radiation efficacy. ECI301 also inhibited growth of other syngenic tumor grafts, including MethA fibrosarcoma (BALB/c) and Lewis lung carcinoma (C57BL/6). Importantly, tumor growth at the nonirradiated site was inhibited, indicating that ECI301 potentiated the abscopal effect of radiation. This abscopal effect observed in BALB/c and C57BL/6 mice was tumor-type independent. Leukocyte depletion studies suggest that CD8+ and CD4+ lymphocytes and NK1.1 cells were involved.

Conclusions: Marked inhibition of tumor growth at the irradiated site, with complete tumor eradication and consistent induction of the abscopal effect, was potentiated by i.v. administration of ECI301. The results of this study may offer a new concept for cancer therapy, namely chemokine administration after local irradiation, leading to development of novel therapeutics for the treatment of advanced metastatic cancer.

Radiation therapy is widely used for many malignant tumors, mostly for local control where a curative or palliative outcome is the intent or as preoperative or postoperative treatment as neoadjuvant or adjuvant therapy. It is also common to use radiation therapy alongside hormone therapy or chemotherapy. To enhance the therapeutic efficacy of radiation sufficiently, we have chosen macrophage inflammatory protein-1α (MIP-1α) as a combination therapy and investigated whether MIP-1α could cause a broad-spectrum enhancement of radiotherapy efficacy in tumor-bearing mice. Radiation treatment at tumor-bearing sites is known to induce inflammation in the irradiated field and to recruit tumor-specific T lymphocytes and dendritic cells, which seem to play an important role in remission of tumors (1–3). MIP-1α or CCL3 is a chemokine known to be secreted from various leukocytes, including T lymphocytes and activated macrophages, and to recruit CCR1-expressing and/or CCR5-expressing leukocytes, such as monocytes, dendritic cells, natural killer cells and T lymphocytes (4). It was also reported that MIP-1α could enhance survival of dendritic cells (5) and primed T lymphocytes to generate IFN-γ (6).

Naive MIP-1α has a known tendency to form large multimeric aggregates. However, an active variant of human MIP-1α with improved pharmaceutical properties that carries a single amino-acid substitution of the 26Asp to Ala was reported (7). This variant, named BB-10010, has a reduced tendency to form large aggregates at physiologic pH and ionic strength, and its recombinant formulation was produced using a budding yeast expression system (8) in a GMP-certified production site. The biological activity of this recombinant protein was confirmed in receptor binding, calcium mobilization, and biological assays in vitro and in vivo (4). Its myelosuppressive effect (9–12) was investigated in several clinical trials of patients receiving chemotherapy (13–16). However, the myeloprotective effect was therapeutically insufficient, and hence, this chemokine has not been developed for cancer therapy.
In this paper, we show that the recombinant MIP-1α variant, now called ECI301, with the same amino acid sequence as BB-10010 but was generated using a fission yeast (Schizosaccharomyces pombe) expression system (17), strikingly enhances the antitumor efficacy of subcutaneous tumor irradiation. Because ionizing radiation sometimes, although rarely, reduces tumor growth distant from the irradiated site, a phenomenon known as the abscopal effect (18–20), we also studied the effect of ECI301 on the growth of tumors at the nonirradiated sites and, thus, its effect on enhancement of the abscopal effect. Our results indicate that the abscopal effect was consistently induced upon ECI301 administration after local irradiation at a tumor site.

Materials and Methods

Animals. Seven-week-old female C57BL/6 and male BALB/c mice were purchased from Nippon SLC and housed in a barrier system with controlled light (12-h light:12-h dark) and temperature (22 ± 2°C). Animals were fed a diet of mouse chow and water ad libitum. All animal experiments were carried out in accordance with the guidelines for animal experiment at the University of Tokyo.

Preparation of chemokine. ECI301, a product of Effector Cell Institute, Inc., is a 69–amino acid variant of human MIP-1α carrying a single–amino acid substitution of Asp26 to Ala, the same primary structure as that of BB-10010 (4). Its recombinant protein was generated using a S. pombe expression system (Asashi Glass Co. Ltd.; ref. 17) and purified to homogeneity. Specific activities for chemotaxis and calcium mobilization were equivalent to those of MIP-1α obtained using a Brevibacillus choshinensis expression system (Protein Express). No glycosylation was found in ECI301.

Tumors and animal models. Lewis lung carcinoma (LLC) and MethA fibrosarcoma cells were obtained from the American Type Culture Collection, and Colon26 adenocarcinoma cells were provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University. These cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and cultured at 37°C under humidified 5% CO2 atmosphere. The Colon38 tumor cell line was kindly provided by Professor Hiroshi Maeda, Faculty of Pharmacology, Sojo University. Colon26 cells (2 × 10⁵ per mouse) and MethA cells (2 × 10⁵ per mouse) were implanted s.c. in the right or both flanks of BALB/c mice depending on the experiments. LLC cells (4 × 10⁵ mouse) were implanted s.c. in the right or both flanks of C57BL/6 mice. Before tumor implantation, cells were trypsinized and filtered through a 70-μm pore size cell strainer. A fragment (~2 × 2 × 2 mm) of Colon38 excised from a tumor-bearing mouse was implanted s.c. in C57BL/6 mice. The tumor volume was calculated using the formula:

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\text{tumor volume} = (\text{major axis}) \times (\text{minor axis})^2 \times 0.5236.
\]

Tumor irradiation and ECI301 administration. Mice-bearing tumors in either right or both flanks (~10 mm diameter) were held in the decubitus position, and ionizing radiation (6 MeV electron beam) was delivered to the right flank from just above the tumor. To cover the target volume of the tumor homogeneously, the reference point for the prescription dose was set at 10-mm depth from the skin surface using the 5-mm tissue-equivalent bolus. The irradiated field was determined using computer tomography–based simulation (Fig. 1) set to exclude hematopoietic organs and critical organs for the immune system. The absorbed dose into the dorsal spine was calculated to be below 10%. Optimum radiation dose was determined using LLC/C57BL/6 combination to be 6 Gy, whereas single 2 or 10 Gy doses were too weak or too strong, respectively, to show the effect of ECI301. After local irradiation, ECI301 was given i.v. beginning at day 1 after irradiation.

Immunohistologic analysis. Tumors were excised 3 days after irradiation (2 days after ECI301 administration), embedded in Tissue-Tek OCT compound (Sakura Fintech, Co., Ltd.), and frozen. Sections were cut to thickness of 6 μm and mounted onto silanized slides (Dako Japan, Inc.). After blocking of nonspecific staining and endogenous peroxidase activity, sections were incubated with predetermined optimal concentrations of the following primary antibodies: antimouse CD4 (L3T4; eBioscience), CD8 (53-6.7; Becton Dickinson), CD45RA (N418; PharMingen), or isotype-matched IgG. The sections were incubated with antirat IgG antibody conjugated with horseradish peroxidase (BIOSOURCE) or goat anti–hamster IgG (H+L) conjugated with alkaline phosphatase (for CD11c, CEDARLANE) and applied with Vector blue (for CD11c; Vector Laboratories) or simple stain 3,3′-diaminobenzidine reagent (Nichirei). The number of immunostained cells was determined by counting under light microscopy at magnification of 400×.

Fig. 1. Field of radiation (left) and computer tomography simulation of the irradiated field (right). The absorbed dose into dorsal spine is below 10%.
Immune cell subset depletion in vivo. The monoclonal rat anti-mouse CD4 (clone GK1.5; ref. 21), rat anti-mouse CD8 (clone 53-6.7; ref. 22), and rat anti-mouse NK1.1 (clone PK136; ref. 23) antibodies were used for immunodepletion. These antibodies were obtained as ascites from hybridoma-bearing nude mice and purified on protein G columns. In the leukocyte depletion studies (21, 22), 100 μg of anti-CD4 or CD8 antibody were injected i.p. to BALB/c mice that carried Colon26 solid tumors at both flanks on the day of irradiation and 4 days after irradiation. Percentage of CD4+ cells in the mononuclear cell fractions obtained from the spleen, the peripheral blood, and the tumor in tumor-bearing mice after (before) anti-CD4 antibody treatment were 0 (14.8 ± 0.9), 0 (9.6 ± 1.3), and 0 (5.3 ± 1.0), respectively, and of CD8+ cells were 5.2 ± 0.8 (6.1 ± 0.5), 7.9 ± 4.1 (3.9 ± 0.6), and 25.9 ± 2.2 (16.8 ± 6.0), respectively. Percentage of CD8+ cells in the same fractions after (before) anti-CD8 antibody treatment were 0.1 ± 0.1 (6.1 ± 0.5), 0.1 ± 0.1 (3.9 ± 0.6), and 0.5 ± 0.3 (16.8 ± 6.0), respectively, and of CD4+ cells were 10.2 ± 2.7 (14.8 ± 0.9), 18.8 ± 9.4 (9.6 ± 1.3), and 11.8 ± 3.4 (5.3 ± 1.0), respectively. For depletion of NK1.1+ cells (23, 24), 100 μg of anti-NK1.1 antibody was i.p. injected to LLC-bearing C57BL/6 mice on the day of irradiation and again 4 days after irradiation. The percentage of NK1.1+ cells in the mononuclear cell fractions from the spleen, the peripheral blood, and the tumor in tumor-bearing mice after (and before) antibody treatment were 0.2 ± 0.2 (1.3 ± 0.2), 0.6 ± 0.3 (15.4 ± 1.2), and 0.4 (8.6 ± 1.1), respectively.

Statistical analysis. Unless otherwise stated, data are presented as mean ± 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 paired Student’s t test. Comparisons among groups in the survival data were made using the log-rank test after Kaplan-Meier analysis. A probability value of P < 0.05 was considered significant.

Results

Effect of ECI301 on tumor growth in mice treated with local irradiation. To determine whether i.v. administration of ECI301 could enhance the antitumor effect of local ionizing radiation, BALB/c mice were implanted with Colon26 adenocarcinoma cells (2 × 10^5 per mouse) in the right flank. When tumor size reached ~10 mm diameter, local irradiation with 6 Gy was delivered to the tumor. After 20 h, i.v. administration of ECI301 (2 μg per mice) was started daily for 5 consecutive days. Tumor growth in ECI301 recipients after local irradiation was inhibited more in animals treated with radiation alone (Fig. 2A), and at 16 days after irradiation, tumor size was reduced by 90% versus 40% for radiation treatment alone (Fig. 2B). Furthermore, the tumor was completely eradicated in three of eight mice that...
received the combination of ECI301 and radiation (Fig. 2C). Similar results were obtained with administration of ECI301 for 3 consecutive days, and in this case also, the tumor was completely eradicated from half of the mice tested (three of six). The optimal dose of ECI301 was determined to be 2 µg per mouse (data not shown). Administration of 2 µg per mouse ECI301 without irradiation caused no noticeable effect with the volume of Colon26 tumors increasing at a similar rate to those in animals receiving no treatment. Combination treatment resulted in tumor-free long-term survival of 38% of mice without significant toxicity (Fig. 2D). When the surviving mice were rechallenged with the same tumor cells (1-2 × 10⁶) subcutaneously at the left or right flank 3 weeks after completion of the combination treatment (a total of 6 weeks after irradiation), no tumor growth was observed at up to 20 days after rechallenge, indicating that the rechallenged tumor cells had been rejected.

Significant, although weaker, enhancement of antitumor radiation efficacy was also observed by weekly administration of ECI301 (Fig. 2A and B). The enhancement of the antitumor effect of local irradiation by ECI301 was also observed with different combinations of tumor type and mouse strains, e.g., LLC cells implanted in C57BL/6 mice (data not shown).

**Effect of ECI301 on tumor growth at the nonirradiated site (the abscopal effect).** Ionizing radiation sometimes inhibits tumor growth outside the field of radiation, a phenomenon known as the abscopal effect (18–20). We investigated whether ECI301 could affect the growth of tumors at a nonirradiated site. Colon26 cells were implanted in the right and left flanks of BALB/c mice and given radiation treatment in the right side only to investigate the effect outside the irradiated field. ECI301 was given at 1, 8, and 15 d starting 20 h after irradiation. Results of mice with radiation treatment only (○) or without treatment (●) were used as controls. Points, mean tumor volume; bars, SE (n = 8). Inset, mean tumor volumes of eight mice 12 d after irradiation; bars, SE. Significant differences from control group: *, P < 0.05; **, P < 0.01 (ANOVA).

| Fig. 3. | Effect of ECI301 administration on Colon26 or LLC tumor growth at the irradiated (B and D) and nonirradiated sites (A and C). A and B, time course of tumor growth. Colon26 cells were inoculated in the left (1 × 10⁵ cells; A) or the right flanks (2 × 10⁶ cells; B) of BALB/c mice and 18 d after inoculation; only the right side was exposed to radiation. ECI301 was given at 1, 8, and 15 d starting 20 h after irradiation (●). Mice with radiation treatment only (○) or without treatment (●) served controls. Significant differences from the control group: *, P < 0.05; **, P < 0.01 (ANOVA). C and D, time course of tumor growth. LLC cells were inoculated in the right (4 × 10⁵ cells; D) and left flanks (2 × 10⁶ cells; C) of C57BL/6 mice and 18 d after inoculation, only the right side was exposed to radiation. ECI301 (▲, 0.08; △, 0.4; ◆, 2 µg per mouse) was given at 1, 8, and 15 d starting 20 h after irradiation. Results of mice with radiation treatment only (○), without treatment (●), or ECI301 administration (2 µg per mouse) without irradiation (●) were used as controls. Points, mean tumor volume; bars, SE (n = 8). Inset, mean tumor volumes of eight mice 12 d after irradiation; bars, SE. Significant differences from control group: *, P < 0.05; **, P < 0.01 (ANOVA). |
These results indicate that induction of the abscopal effect by ECI301 is not restricted to tumor type nor to mouse strain.

**Inhibition of heterotypic tumor growth at the nonirradiated site.** We next investigated whether the abscopal effect is observed in heterotypic tumors grown in the same mouse. In these experiments, we used the combination of MethA fibrosarcoma and Colon26 cells transplanted on the left and right flanks, respectively, in BALB/c mice and that of Colon 38 tissue block and LLC cells on the left and right flanks, respectively, in C57BL/6 mice, with only the right side of each tumor (Colon26 or LLC) exposed to ionizing radiation in both systems. Mice received ECI301 i.v. at 1, 8, and 15 days after irradiation. As shown in Fig. 4, the ECI301-induced abscopal effect was not tumor-selective and the growth of tumors at the nonirradiated site was significantly inhibited in all tumor combinations tested.

Using Colon26 as a model, we investigated whether local irradiation of normal tissues induces the abscopal effect. The tumor cells were implanted in the left flank of BALB/c mice, and radiation (6 Gy) was delivered to the right hind flank of the tumor-bearing mice when the implanted tumor reached ~10 mm in diameter. Irradiation on the normal hind flanks of mice did not induce the antitumor effect, and administration of ECI301 did not influence the results (data not shown). The result indicates that the ECI301-induced abscopal effect depends on irradiation of the primary tumor, indicating that the antitumor effect of ECI301 may be exerted via inflammation and immune response in irradiated tumor tissue.

**Infiltration of CD4+ and CD8+ cells to the tumor-growing site.** We investigated leukocyte infiltration into the tumor sites after combination treatment in LLC-bearing C57BL/6 mice. Two days after ECI301 administration, marked infiltration of CD4+ and CD8+ cells, respectively, were observed not only in the tumor at the irradiated site (typically, 35 and 16 cells per section), but also at the nonirradiated site (58 and 20 cells per section). This effect was not apparent after the administration of ECI301 without irradiation (15 and 8 cells per section), after irradiation alone (11 and 8 cells per section), or no treatment (11 and 4 cells per section) in the tumor at the right flank (Fig. 5). It was reported that dendritic cell precursors were mobilized into the circulation by administration of MIP-1α (25). However, we did not observe an increase in CD11c+ cell infiltration into the tumor tissue in this model (data not shown).

![Fig. 4](image-url)
Immune cell requirement for combination effect. To examine the contribution of specific lymphocyte subsets to the enhancement of anticancer radiation efficacy and the abscopal effect, we used Colon26 cells grown subcutaneously in BALB/c mice and LLC cells in C57BL/c mice and did leukocyte depletion studies using antibodies against cell-surface markers for CD4, CD8, and NK1.1. More than 90% of CD4+ T cells, CD8+ T cells, and NK1.1+ cells were depleted in spleen cells and peripheral blood mononuclear cells of either strain of mice. In the Colon26/BALB/c system, depletion of CD8+ cells reversed the suppressive effect of ECI301 on the right (the irradiated site) and the left flanks (the nonirradiated site) markedly, although the latter was not statistically significant (Fig. 6, top). On the other hand, depletion of CD4+ cells abrogated the suppression of tumor growth at the nonirradiated side, whereas the irradiated site was affected only slightly (Fig. 6, middle). The injection of isotypes of these antibodies did not affect the suppression effect by ECI301 (data not shown).

As shown in Fig. 6 (bottom), depletion of NK1.1 cells in LLC-bearing C57BL/6 mice reversed the abscopal effect by ECI301 and the tumor size at the nonirradiated site became similar to that without administration of ECI301. In contrast, the growth of tumors at the irradiated site at the right flank was unaffected. Finally, we tested whether depletion of natural killer cells by anti-asialo GM1 antibody could affect the antitumor radiation efficacy and the abscopal effect by ECI301. Although this treatment efficiently, but not perfectly, depleted the natural killer cells in peripheral blood mononuclear cells and spleen cells of the animals, the suppressive effect of tumor growth at both flanks by ECI301 was not affected (data not shown).

Discussion

In this study, we show that a combination of radiotherapy and ECI301 i.v. administration effectively prevented tumor growth at the irradiated site. Prevention of tumor growth was observed with various mouse tumors, including LLC, MethA fibrosarcoma, and Colon26 adenocarcinoma, suggesting that the effect was not restricted to a specific tumor type. A three to five times of repeated daily administration after local irradiation at the tumor growing site resulted in complete remission of about half of the mice treated. When the same tumor, but not a different one, was inoculated again to the cured mice, no tumor regrowth was observed. Repeated weekly administration of ECI301 after irradiation also gave significant, but less effective, results, suggesting that, for optimum ECI301 efficacy, repeat administration within a 7-day window after radiation treatment is required. No notable toxicity was found in mice as a result of the i.v. administration of ECI301. In accordance with this finding, no significant side effects of a compound with the same structure...
Enhanced Radiation Efficacy and Induced Abscopal Effect

Although there are many reports concerning anticancer (26–31) and antimetastasis effects of MIP-1α (32), enhancement of radiation efficacy has not been investigated previously. Local ionizing radiation not only induces necrosis and apoptosis of cells due to DNA damage but may also cause inflammation with concomitant recruitment of leukocytes due to chemical mediator production (33). Ionizing radiation has been reported to induce recruitment of T-helper cells and cytotoxic T cells (2, 14, 34, 35). It is possible that ECI301 activates lymphocytes and that the chemotactic property of ECI301, adsorbing to tumor sites, led to an enhancement of the radiation-induced recruitment of lymphocytes. Histologically, CD4+ and CD8+ T cells were detected in the irradiated tumors (and in non-irradiated tumors of the same mice). Recruitment was not apparent with radiation alone and less apparent with ECI301 alone. Depletion of CD8+ T cells by antibodies diminished the effect of combination treatment at the irradiated site, indicating that CD8+ T cells are involved in the antitumor effect. Furthermore, rejection of the same tumor type in the cured mice may be mediated by the presence of these types of lymphocytes. Increased number of splenocytes with tumor-specific IFN-γ generating ability by combination treatment also support this assumption.

By the administration of ECI301 after local radiation treatment to a tumor-bearing area, systemic effects were observed and tumor growth distant to the irradiated site was prevented. This suggests that combination treatment of radiation and ECI301 has the potential to control metastatic tumors. Radiation treatment to a local area of the body sometimes results in an antitumor effect at another location in the body as in the case of a Japanese patient who was reported with hepatocellular carcinoma that regressed after palliative local radiotherapy for pain control of bone metastases (36). This rare phenomenon is known as the abscopal effect and defined as a reaction produced after irradiation but occurring outside the zone of actual radiation absorption (18–20). In the present study, the abscopal effect was not detectable without administration of ECI301 or when normal tissues received radiation. This is in contrast to the report of Camphausen et al., who showed that irradiation of normal tissue induced the abscopal effect and concluded that p53 rather than an immune mechanism was responsible (37). In their study, however, more severe radiation regimens (10 Gy for 5 consecutive days and 2 Gy for 12 consecutive days) were used than in the present studies. The abscopal effect enhanced by ECI301 required a single 6-Gy fraction or 2 Gy for 5 consecutive days (results not shown), which is close to a clinically relevant regimen, with animal appearing healthy after irradiation.

Demaria et al. reported that the abscopal effect was immunemediated and T cell–dependent, using a syngeneic mammary carcinoma 67NR by radiation together with Flt3-ligand, the growth factor for dendritic cells (38). In the present study, CD8+ cells seem to be involved in the ECI301-provoked abscopal effect because depletion of these leukocytes partially reversed the effect. However, because the abscopal effect was also observed for heterotypic tumors, a broad spectrum antitumor immune response is stimulated. Depletion of CD4+ T lymphocytes or NK1.1 cells by an antibody treatment diminished the abscopal effect, indicating that these cells are involved in the remission either directly or indirectly. CD4+ T cells may play a role in generating cytokines, such as IFN-γ, which may also activate other leukocytes (3, 39, 40).

Finally, soon after local tumor irradiation, tumor growth at the irradiated site was reduced, and this effect was enhanced by ECI301 administration (see Figs. 2–4). This response is too rapid to attribute to an antigen-dependent immune response. Although the lymphocyte depletion test showed that CD8+ lymphocytes were involved in the increased radiation efficacy, there may be another CD8+ cell-independent effect of ECI301, which also seems to be involved in the abscopal effect (see Figs. 3 and 4). Further studies are required to clarify this point. In experiments not shown in this paper, we found various antitumor chemotherapeutic agents, such as docetaxel, diminished the effect of ECI301 when injected before administration

Fig. 6. Effect of CD8+ or CD4+ T lymphocyte or NK1.1 cell depletion on tumor growth in mice that received combination therapy. CD8+ (top) or CD4+ T lymphocytes (middle) were depleted by the antibodies from BALB/c mice bearing Colon26 tumor at both flanks. NK1.1 cells (bottom) were immunodepleted from C57BL/6 mice bearing LLC tumor at both flanks. No depletion study of NK1.1 cells was done for control and radiation treatment. Each antibody was injected i.p. on the day of irradiation and 4 d after irradiation. ECI301 was given 20 h after irradiation. Columns, mean tumor volume of eight mice at day 9 after irradiation. Significant differences between the nontreatment and specific antibody-treated groups: \( P < 0.05; \), \( P < 0.01 \) (Student’s t test). Left and right of each pair of columns show results of nontreatment and specific antibody-treated groups, respectively.

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4 Unpublished observation.
of EC1301. The recruitment of leukocytes may have been inhibited by the antitumor chemotherapeutic agents, which would support the assumption that some kind of recruited leukocytes play a role in the enhancement of radiation efficacy and the abscopal effect.

In conclusion, we have presented data that support a new concept for cancer therapy, i.e., chemokine administration after radiation treatment, and showed its effectiveness in animal studies with demonstrable systemic effects. These data will encourage future therapeutic application of EC1301 in the treatment of advanced metastatic cancer.

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References
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