Combination of direct intratumoral administration of dendritic cells and irradiation induces strong systemic antitumor effect mediated by GRP94/gp96 against squamous cell carcinoma in mice

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Abstract. We tested a new therapeutic modality for head and neck and esophageal cancers, a combination of direct intratumoral (i.t.) administration of dendritic cells (DCs) and radiation therapy (RT) in mouse squamous cell carcinoma (SCC). We also evaluated the functions of gp96, which can enhance systemic antitumor activity, and the mechanism of the abscopal effect. Mouse SCC cells ($1x10^5$), SCCVII, were inoculated into the left femur of C3H/He mice subcutaneously, and also similarly inoculated into chest subcutaneous tissue. Only the left femur tumor was exposed to 4 or 10 Gy of ionizing radiation, and then $1x10^6$ DCs i.t. was injected only into the femur tumor. Following this procedure, tumor volumes of the femur and chest were measured. We evaluated whether gp96 could enhance the antitumor effect. With DCs i.t. and RT, tumor growth was markedly suppressed. Tumor growth of non-treated tumors were also suppressed, indicating that the combination therapy of DCs and RT evoked systemic antitumor activity. *In vitro*, the enhancement of gp96 expression was strongly detected by immunostaining after irradiation, DCs with gp96 induced strong cytotoxic activity *in vitro*, and tumor growth inhibition was observed by direct i.t. injection of gp96. A combination of DCs i.t. and RT can induce a strong antitumor effect not only against treated local tumor but also against non-treated distant tumor, indicating that this treatment can evoke a strong systemic antitumor effect. Gp96 is thought to be one of the target molecules to explain the abscopal effect.

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Introduction

Despite the recent advances in treatment, head and neck cancer and esophageal SCC (squamous cell carcinoma) are still among the worst malignant neoplasms, especially in Asia. Their prognosis is miserable and unsatisfactory with the current treatments, as lymph node metastases and distant organ metastases occur frequently. Effective treatment is not yet established, and new therapeutic modalities are eagerly awaited.

DC (dendritic cell)-based immunotherapy is reported as an effective treatment in many types of cancers, but the usage of DCs alone is not always completely effective because of the poor antigenicity of cancer cells themselves. The precise reason why cancer can escape from the immune system of the host is still unclear. In recent years, various possibilities of combining immunotherapy and RT (radiation therapy) that would be able to achieve better local tumor control and systemic tumor control (1-5) have been discussed. RT has the ability to kill cancer cells, and RT-killed cancer cells can be a good source of tumor antigens for inducing CTL (cytotoxic T-lymphocyte) activation (6). Moreover, a recent study revealed that RT itself can elicit a systemic immunological antitumor effect (7) called the abscopal effect (8). However, direct evidence on how ionizing radiation can elicit a systemic antitumor immunoresponse is not available.

Heat shock protein gp96, also called glucose-regulated protein GRP94, is a stress protein that works as protein chaperone when DCs take up the antigen via surface molecule CD91 (9-12) to mediate CTL activation by Toll-like receptors 2 and 4 (13). We think that gp96 is a key molecule for explaining the mechanism of the abscopal effect. In this study, we evaluated the expression of gp96 induced by ionizing radiation and the function of gp96 for the enhancement of the immunoresponse both *in vitro* and *in vivo*. We present the usefulness of the combination therapy of direct DCs i.t. (intratumoral) and RT, which can induce strong antitumor effect not only against local but also distant non-treated tumor, using a mouse SCC model. This therapy gives hope to patients with head and neck and esophageal cancers.

Materials and methods

Tumor and mice. Poorly immunogenic, mouse squamous cell carcinoma SCCVII tumor cells, kindly provided by Professor Yuta Shibamoto (Department of Quantum Radiology, Nagoya City University, Nagoya, Japan), were used in this study. The characteristics of these tumor cells have been well described (14). SCCVII cells were maintained in Eagle's minimum essential medium supplemented with 12.5% fetal bovine serum, penicillin and streptomycin (100 μg/ml), and 2-[4-(2-Hydroxyethyl)-1-piperadinyl] ethansulfonic acid (10 mM). Syngeneic 6- to 10-week-old female C3H/He mice, purchased from Japan SLC (Shizuoka, Japan), were maintained in our facility under specific pathogen-free conditions. Independent experimental groups were used, and each consisted of at least 3 mice. Animal care was in accordance with the guidelines of Chiba University.

DCs generation. DCs were generated using the method established by Lutz *et al* (15). Cells were prepared from BM (bone marrow) cells of femurs and tibias. First, BM cells were left in 70% ethanol for 2-5 min for disinfection, washed with PBS (phosphate-buffered saline) 2 times, and then flushed with PBS using a 27-gauge injection needle. On day 0, $2x10^6$ BM cells in 10 ml of RPMI-1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), 2-mercaptoethanol (50 μM), 10% fetal bovine albumin, and 200 U/ml (20 ng/ml) rmGM-CSF (recombinant mouse granulocyte-colony stimulating factor) (G0282, Sigma, Tokyo, Japan) were placed into 100-mm diameter Petri dishes (Falcon™ No. 1029, BD, San Jose, CA, USA). On day 3, another 10 ml of the medium with supplements was added to the dishes. Non-adherent cells were harvested on day 6 for use as DCs.

Flow cytometry. Cells were first treated with mouse BD Fc Block (2.4G2, Pharmingen™, BD) and then stained with antibodies conjugated with fluorescent agents. For analysis of the surface markers of DCs, anti-mouse CD11C-FITC (BD-557400, BD), anti-mouse CD80-PE (BD-553769, BD), anti-mouse CD86-PE (BD-553692, BD) and anti-mouse I-Ab (BD-553605, BD) were used. For the investigation of CTLs in TDLN (tumor drainage lymph node)s, CD3-PE (2134-0034, Biogenesis, Kingston, NH, USA) and CD8-FITC (2134-0083, Biogenesis) were used. Cell count was performed with a Coulter Epics XL cytometer (Beckman Coulter, Miami, FL, USA), and cell populations were evaluated with gating software, FlowJo for Windows (Tree Star Inc., Ashland, OR, USA).

Fluorescent staining of gp96 in SCCVII cells in vitro. SCCVII cells ($1x10^6$) were exposed to 2 Gy of ionizing radiation. Twenty-four hours later, cells were collected and stained with anti-mouse-gp96 (SPA-850, Stressgen, Ann Arbor, MI, USA) and fluorescein-conjugated secondary antibody (Chemicon, Temecula, CA, USA). The expression of gp96 was observed with a laser microscope (Carl Zeiss, Oberkochen, Germany).

Western blot analyses. Protein extracts were separated by electrophoresis on 10% polyacrylamide gel. The proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA) in a tank transfer apparatus (Bio-Rad, Hercules, CA, USA), and the membranes were blocked with 5% skim-milk in PBS. Rat anti-GRP94 monoclonal antibody (SPA-850, Stressgen) diluted to 1:2000 in blocking buffer was used as primary antibody, and goat anti-rat IgG: horseradish peroxidase Conj (SAB-200, Stressgen) diluted to 1:5000 in blocking buffer was used as secondary antibody. Antigens on the membrane were detected by ECL™ detection reagents (Amersham Biosciences, Piscataway, NJ, USA).

Cytotoxic activity mediated by DCs pulsed with/without antigen and with/without gp96. To generate antigen, WTL (whole tumor lysate) was made. Viable SCCVII tumor cells were suspended at $1x10^7$/ml PBS and subjected to four cycles of rapid freeze (liquid N_2)/thaw (37˚C water bath) exposure. Then they were spun at 1000 rpm at 4˚C for 5 min to remove cellular debris, and the supernatant was used as antigen. DCs were mixed with WTL at a ratio of 3 tumor cell equivalents: 1 DC in complete medium or in complete medium including pathogen-free purified gp96 (5 μg/ml) eluted from mouse cells (IMA-200-1-C100, Immatics Biotechnologies, Tübingen, Germany). After 18 h of culture, DCs were harvested, washed twice with PBS, and resuspended at $1x10^6$ cells/0.1 ml PBS for use as TP-DCs (tumor-pulsed DCs) or as gp96-pulsed TP-DCs (gp96-TP-DCs). Next, whole spleen cells of C3H/He mice were mixed with TP-DCs or with gp96-TP-DCs, and incubated for 24 h. As target cells, SCCVII cells were labeled with 100 μCi of sodium chromate (^{51}Cr; Amersham Pharmacia Biotech, Uppsala, Sweden) for 1 h at 37˚C. Then, the mixture of DCs with splenocytes was co-cultured with ^{51}Cr-labeled SCCVII cells for another 4 h. Cytotoxic activities were evaluated at E/T ratios of 100, 50 and 25. ^{51}Cr release assay was conducted as previously described (16).

Tumor growth inhibition by direct i.t. administration of gp96 in vivo. We examined the effectiveness of direct i.t. administration of purified mouse gp96. SCCVII cells were inoculated into femur subcutaneous tissue, and 4 μg of gp96 was directly injected into tumors on days 6, 7 and 8 after the tumor inoculation. Tumors were measured on days 3, 7, 10, 11, 16, 18, 22, 25, 28 and 32, and tumor volumes were calculated.

CTLs in TDLNs. First, SCCVII cells at $1x10^5$/50 μl PBS were inoculated into the left femur subcutaneous tissue of C3H/He mice on day 0. On days 6, 7 and 8, tumors were exposed to 10 Gy of radiation, and then DCs i.t. injection was performed on days 8, 9 and 10. On day 11, mice were sacrificed and TDLNs were collected and washed with PBS. After red blood cell reduction, cells were stained with fluorescent conjugated anti-CD3 and anti-CD8, as described for the aforementioned flow cytometry. The population of $CD3^+/CD8^+$ as a systemic cytotoxic reaction was evaluated.

Direct DCs i.t. with RT. For treated local tumor, SCCVII cells ($1x10^5$ cells/50 μl PBS) were inoculated into the left femur subcutaneous tissue of C3H/He mice on day 0. Similarly, for non-treated distant tumor, SCCVII ($1x10^5$ cells/50 μl PBS) were inoculated into chest subcutaneous tissue. Before

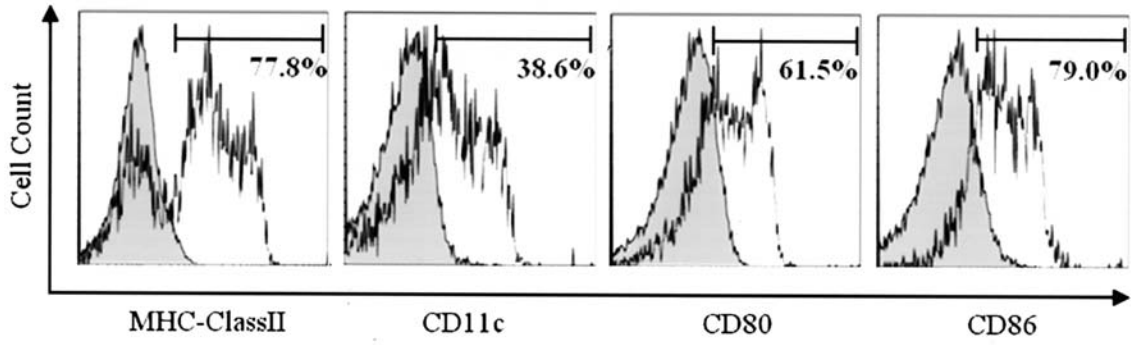

Figure 1. Cell surface phenotypes of BM-derived cells and DCs. BM-derived cells were cultured in the presence of rmGM-CSF and then stained for flow cytometry. Cultured cells presented high levels of MHC-class II, CD11c, CD80 and CD86 molecules (white) compared with BM-derived cells (gray).

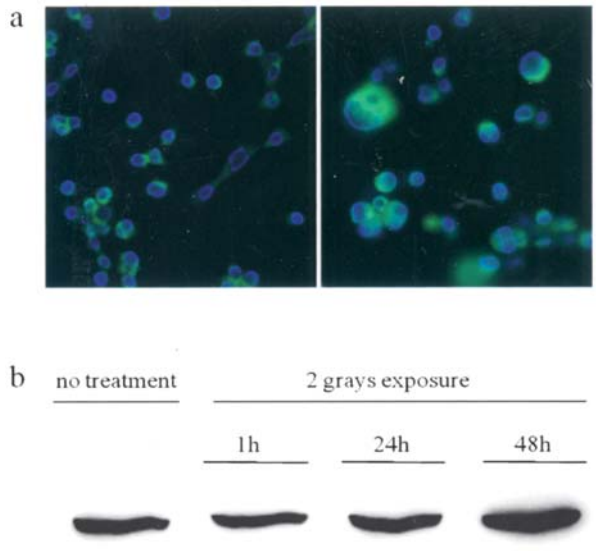

Figure 2. a, Fluorescent staining with anti-gp96-FITC antibody 24 h after exposure to 2 Gy of radiation. Compared with normal control (left), gp96 was well stained in tumor cells mainly at the cytoplasm, indicating that ionizing irradiation induced enhancement of gp96 expression (right). Cell ballooning was also observed in irradiated cells. b, Western blotting of gp96. SCCVII tumor cells were exposed to 2 Gy of ionizing radiation and analyzed by Western blotting 1, 24 and 48 h after the exposure. Gp96 protein was increased at 48 h after the irradiation compared with normal control.

DCs i.t., 4 Gy of ionizing irradiation was given only to the femur tumor on days 6, 7 and 8 after tumor inoculation. To avoid systemic exposure by RT, the whole body except the femur tumor was covered with a lead shield. On day 8, 9, 10, 12 and 14, $1x10^6$ DCs were directly administered only into the femur tumor. Both femur and chest tumors were measured on days 3, 6, 8, 10, 12, 14, 16, 18, 21, 24, 29, 33 and 38, and tumor volumes were calculated.

Results

Surface markers of generated DCs. Because DCs generation according to Lutz *et al* (15) was a newly developed method, we first evaluated whether cells thus generated could be used as DCs. The surface markers of these cells treated with this technique are shown in Fig. 1. MHC (major histocompatibility complex) class II, CD11c, CD80 and CD86 were highly expressed at 77.8, 38.6, 61.5 and 79.0%, respectively. Accordingly, we used these cells as DCs.

Induction of gp96 by ionizing radiation, and cell ballooning. We examined whether the expression of gp96 could be induced by irradiation *in vitro*. When SCCVII cells were exposed to 2 Gy of irradiation, the induction of gp96 protein was detected especially in the cytoplasm by fluorescent staining as shown in Fig. 2a. Similarly, enhanced expression of gp96 was detected by Western blotting 48 h after irradiation (Fig. 2b). These results indicate that the expression of gp96 was stimulated by the stress of the irradiation. Additionally, cell ballooning was observed by laser microscopy.

Gp96 enhancement of cytotoxic effect in vitro and in vivo. We next evaluated whether gp96 could induce the activation of cytotoxic activity *in vitro*. As shown in Fig. 3a, the cell mixture of DCs, WTL, splenocytes and gp96 induced the strongest cytotoxicity against ^{51}Cr-labeled SCCVII cells compared with the mixture without gp96. This implies that gp96 itself has the ability to stimulate DCs. Furthermore, DCs, gp96, and splenocytes, but without WTL, also induced cytotoxicity. Gp96 itself, without the need to identify specific tumor antigenic epitopes, could induce cytotoxicity, and can induce the maturation of DCs and elicit antitumor responses (17), whereas simple co-culture of DCs and splenocytes could not. Then, we evaluated the effectiveness of gp96 *in vivo*. As shown in Fig. 3b, slight growth suppression was observed when gp96 was i.t. administered. This indicated that gp96 itself can induce tumor suppression, which seems to be mediated by the DC-based immunoresponse as demonstrated in Fig. 3a.

Population of $CD3^+/CD8^+$ T cells in TDLNs. We investigated whether DCs i.t. can elicit a systemic antitumor effect *in vivo* by detecting $CD3^+/CD8^+$ T cells in TDLNs. As shown in Fig. 4, the $CD3^+CD8^+$ population in TDLNs of the mice treated by both DCs i.t. and RT was about 2 times larger compared with those treated only with DCs i.t. This suggests that RT can effectively evoke systemic antitumor activity and that it can be a sensitizer of a DC-based immunoresponse. Furthermore, we also evaluated whether RT treatment alone has the possibility of inducing $CD3^+CD8^+$ T cells, and it was found

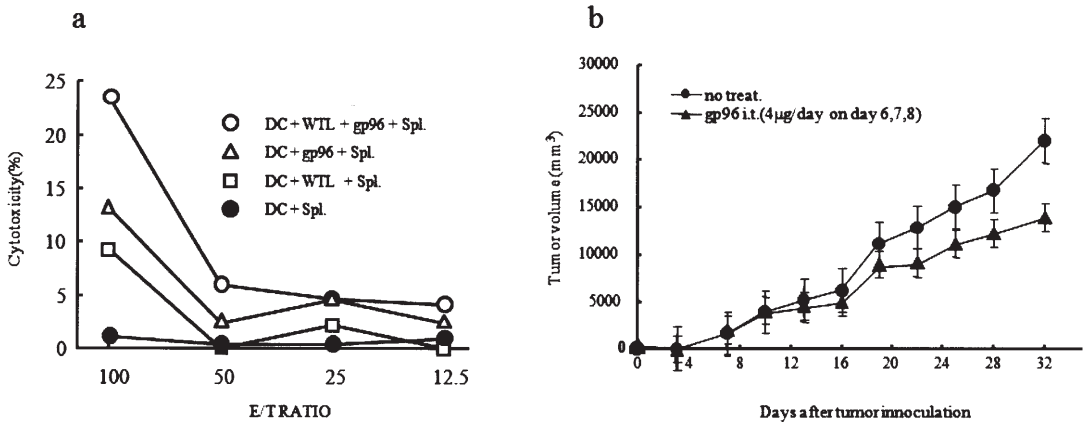

Figure 3. a, Cytotoxicity of mixture of DCs and splenocytes primed with/without WTL and with/without gp96 *in vivo*. DCs pulsed with both WTL and gp96 showed enhanced antitumor effect (open circles) compared with DCs pulsed only with WTL (open squares). DCs pulsed without WTL did not induce cytotoxicity in this examination. Surprisingly, DCs pulsed only with gp96 also showed enhanced cytotoxicity (open triangles). b, Tumor growth inhibition by gp96 *in vivo*. SCCVII tumor cells ($1x10^5$) were inoculated into femur subcutaneous tissue on day 0. Four micrograms of gp96 was directly administered into tumors 6, 7, and 8 days after the inoculation of tumors. Suppression of tumor growth was observed (closed triangles), although complete remission was not observed. Bars ± SE.

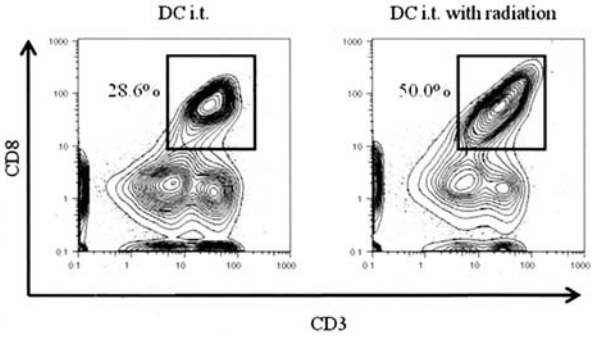

Figure 4. Ionizing radiation can raise $CD3^+CD8^+$ T cells in TDLNs, *in vivo*. SCCVII cells ($1x10^5$) were inoculated, and on days 8, 9 and 10, $1x10^6$ DCs were directly injected into the left femur tumor. Shown is the population of $CD3^+CD8^+$ T cells in TDLNs. The population of $CD3^+CD8^+$ T cells in TDLNs preliminary added with 10 Gy of irradiation on days 6, 7 and 8 is shown on the right. Treatment with DCs only is shown on the left. In mice treated with both DCs i.t. and RT, the population of $CD3^+CD8^+$ T cells was 2 times larger than in those treated only with DCs i.t.

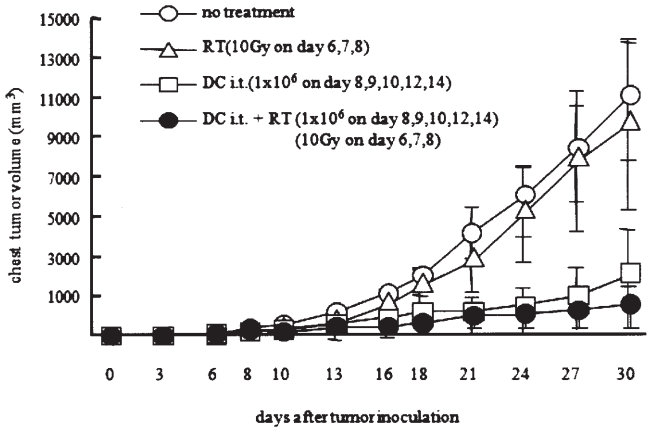

Figure 6. Non-treated tumor growth inhibition mediated by systemic antitumor effect induced by combination of DCs i.t. and RT. SCCVII tumor cells ($1x10^5$) were inoculated both into left femur subcutaneous tissue and into chest subcutaneous tissue on day 0. Only the left tumor was treated with DCs i.t. (on days 8, 9, 10, 12 and 14) and 10 Gy of RT (on days 6, 7 and 8). Closed circles indicate the chest non-treated tumor volume. The left femur tumor was treated with both DCs i.t. and RT. In these mice, distant non-treated tumor growth was markedly suppressed, compared with mice treated only with DCs i.t. (open squares). Bars ± SE.

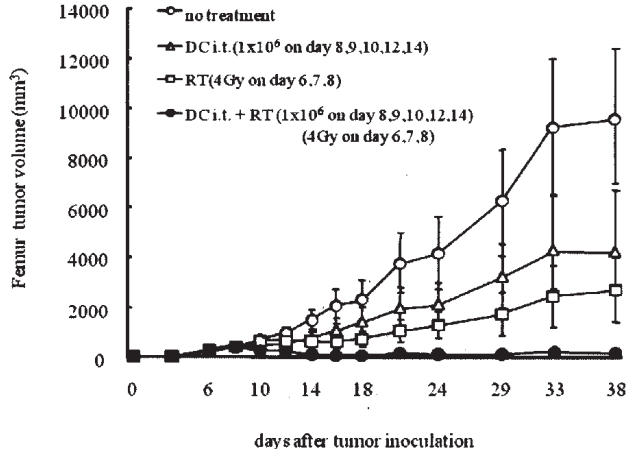

Figure 5. The effectiveness of combination therapy of DCs i.t. and RT. SCCVII tumor cells ($1x10^5$) were inoculated into the left femur subcutaneous tissue. $1x10^6$ DCs i.t. (on days 8, 9, 10, 12 and 14) following 4 Gy of RT (on days 6, 7 and 8) could almost completely suppress tumor growth (closed circles). The rate of tumor growth suppression was 98.6%. Open triangles indicate tumor volume treated only with DCs i.t., and open squares indicate those treated only with RT. Bars ± SE.

that RT alone could indeed induce that population (data not shown). This may be one of the pieces of evidence that will explain the mechanism of the abscopal effect.

Efficacy of combined therapy of DCs i.t and RT against local treated tumor in vivo. We then examined the antitumor effect by the combination of DCs i.t. and RT against local tumor. The femur tumor grew to a mean size of $1.2x10^4$ mm^3 by day 38. The mean inhibitory rate of growth of femur tumors

treated with DCs i.t. was 50.1% compared with control mice. The mean inhibitory rate of tumors treated with RT alone was 72.3% compared with control. The mean inhibitory rate of tumors treated with DCs i.t. and RT was 98.6% compared with control. Thus, the combination therapy of DCs i.t. and RT showed the strongest antitumor effect *in vivo* (Fig. 5).

The efficacy of combined therapy of DCs i.t. and RT against non-treated distant tumor in vivo. We investigated whether the combination of direct DCs i.t. and RT could elicit a systemic antitumor effect. As seen in Fig. 6, the mean inhibitory rate of tumors treated only with DCs i.t. was 74.4%, and that with DCs i.t. and RT was 87.3%, the strongest. With RT only, the inhibitory rate was 10.8% compared with normal control. This phenomenon was thought to be the abscopal effect.

Discussion

In 1953, the abscopal effect, which was defined as a significant tissue response to radiation that occurs beyond the radiation field, was first reported by Mole (8). He proposed the term 'abscopal', derived from the Latin prefix *ab-* 'position away from' and *-scopos* 'mark or target for shooting at'. This mechanism is partly revealed by the fact that ionizing radiation induces the release of cytokines or other inflammatory factors in the circulation (18,19), or that it induces tumor cell death and generates inflammatory signals that mediate direct effects on tumor-associated vessels and also enhance the permeability of solid tumors to both DCs and CTLs (20,21). Recent studies suggested that the mechanism of the abscopal effect is T-cell mediated (7), or that it is mediated by engagement of the Fas/Fas ligand pathway (22), but it still remains poorly understood.

We think one key molecule, by which the abscopal effect induces systemic antitumor immune reaction, is gp96. Gp96 is a major member of the heat shock protein 90 family (23,24) induced by several stresses, resides in the endoplasmic reticulum, and works as a protein chaperone. As seen in Fig. 2, RT has the ability to induce the expression of gp96 in the cytoplasm and also cell ballooning *in vitro*. DCs reportedly acquire antigen both from apoptotic and necrotic cells (25-27) to induce class I-restricted CTLs. However, we cannot conclude that the ballooning of irradiated SCCVII cells will lead to apoptosis or necrosis, and we cannot answer the question of which is the better source of antigens to induce CTLs. Gp96 plays an important role when DCs take up antigens. First, the tumor antigen and gp96 are combined and form gp96-antigen complex. Following this, the complex is taken up by DCs via CD91 (gp96 receptor) expressed on DCs (9-12,28,29) mediated by Toll-like receptor 2 and 4 (13). By these processes, DCs are matured and then induce $CD8^+$ CTL activation (30) by cross-presentation (31), a mechanism equally reported in human (32,33). Our findings demonstrated that DCs pulsed with gp96 could exert a stronger cytotoxic effect *in vitro* and gp96 i.t. could induce slight tumor growth inhibition *in vivo* (Fig. 3).

Overexpressed gp96 by radiation induced $CD8^+$ CTLs in TDLNs, as seen in Fig. 4. As others have reported (29,34,35), gp96 potentially boosts the activity of the systemic antitumor effect by these CTLs, and this mechanism could be one explanation for the abscopal effect. In brief, the amount of gp96 is important, although gp96 exists naturally in ordinary normal cells. On the other hand, our data showed only slight tumor suppression by gp96 i.t., confirming the recent reports that non-tumor-derived gp96 cannot induce strong antitumor immunity *in vivo* (36,37). We used purified gp-96 that was non-tumor derived, so the direct injection of non-tumor-derived gp96 was not sufficient for tumor growth inhibition. This suggests that a stronger antitumor effect might be attained if we used the SCCVII tumor-derived gp96. These data imply that the important factor in inducing the activation of DCs is not only the amount of gp96 but also its source.

DC-based immunotherapy results in significant tumor response in various types of tumors. The administered agents are patient-oriented, meaning that toxic adverse effects would presumably be reduced. In this regard, DC-based immunotherapy is quite gentle for patients. However, it is not always completely effective in clinical trials based on immunotherapy alone, as cancer cells often escape from the immune system of the host. Thus, the major problem of poor antigenicity of the tumor can not be ignored. The precise reason why cancer cells escape from the immune system is still unclear, but poor antigenicity is certainly involved. Several attempts have been made to overcome this problem (38-41). As Teitz-Tennenbaum *et al* reported, ionizing radiation is one of the candidates for potentiating the therapeutic efficacy of DCs i.t. (42). Likewise, this has been supported by Lugade *et al* (43), who showed that RT induces a greater capability of tumor cells to present tumor antigens on their surface and enhance specific T cells in TDLNs. Such combinational treatments have been reported as useful in some other malignancies, such as fibrosarcoma (44), breast cancer (45) and melanoma (42).

SCC is known to possess sensitivity for RT, so that strong efficacy of the combination of DCs i.t. and RT, which can produce tumor antigens by apoptosis and/or necrosis of tumor cells, and also induces the abscopal effect against SCC, can be expected. A second important strategy is direct DCs i.t. This technique has two major benefits. First, it is profitable for antigen up-taking by DCs. DCs i.t. is lucrative because DCs can be pulsed with a wide range of antigens, such as WTL, proteins, peptides, DNA, and RNA. Second, it is efficient for direct trafficking into tumor, and this means that the trafficking loss of DCs administered is minimized (40,45,46). Moreover, a DC-pulsing procedure is thus unnecessary for DCs i.t. DCs i.t with RT could induce stronger antitumor effect against local tumors compared with tumors treated with simply DCs i.t., as our data show in Fig. 5. This point is the most important for cancer treatment, as the combination of DCs i.t. and RT exerted a precise effect against distant non-treated tumors (Fig. 6), meaning that this combination can evoke a strong systemic antitumor reaction thought to be mediated by the abscopal effect.

Head and neck and esophageal cancers are still among the worst, and deadly, malignant neoplasms, especially in Asia. These cancers have the tendency to metastasize easily to lymph nodes (29,47), the major reason for their miserable prognoses, in spite of the recent improvements in early-stage detection (48) or in advanced-stage treatment. The urgency for the development of an effective treatment is very clear.

At present, chemotherapy is the only systemic treatment method for SCC, but it tends to lead to a severe immunosuppressed condition of the patient, and its performance is far from satisfactory, as the highly frequent incidents of adverse events cannot be ignored. Our therapeutic model of DCs i.t.-based immunotherapy with RT presents a useful and effective therapeutic modality not only for local tumor but also systemic metastases. RT is routinely and easily performed, and the direct i.t. technique can also be quite readily performed via endoscope or intervention techniques. DCs i.t. with RT treatment is certain to become a treatment modality with much promise for SCC cancer patients in the near future.

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