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Original Research Article

Identification of the active portion of the CCL3 derivative reported to induce antitumor abscopal effect

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ABSTRACT

Background and purpose: Intravenous administration of a single amino acid-substituted chemokine CCL3 derivative named eMIP elicits the abscopal effect (an effect distal to the target), after local irradiation at a tumor-bearing site. To distinguish the active portion of eMIP, we tested the antitumor activity of chemically synthesized partial peptides of eMIP. Synthetic peptide has various advantages in its clinical application.

Material and methods: Colon26 adenocarcinoma cells were implanted subcutaneously in the right and left flanks of mice. eMIP, CCL3 or any of synthesized peptides was administered intravenously, either after irradiating the right flank. The effect was evaluated by tumor-growth inhibition.

Results: Q/C peptide, a synthetic peptide of amino acids 22–51 of eMIP has no chemotaxis-inducing ability but yet enhanced tumor growth inhibition at the non-irradiated sites, recapitulating the effect of eMIP with local irradiation. Co-administration of this peptide and HSP70 also inhibited tumor growth.

Conclusions: Q/C peptide maps to the eMIP β-sheet: 3 adjacent anti-parallel strands connected by the β-hairpins, is the active portion of eMIP necessary for an immunomodulatory antitumor effect. This experimental reduction furthers our understanding of the underlying mechanism of the abscopal effect. The data will open the way for therapeutic application of like peptides.

Introduction

Inflammation, with concomitant recruitment of leukocytes, induced by local irradiation, is known to play an important role in remission of tumors [1,2] and to affect patient outcome. In rare cases, regression of metastases outside of the radiation field has been reported after local irradiation of one or more of the tumor sites. This phenomenon is called the abscopal effect and mediated by an immune reaction induced by the localized irradiation [3,4]. We previously reported induction of tumor type-independent and -dependent abscopal effect in animal model experiments by administration of a derivative of CCL3 after local irradiation, accompanied by striking tumor growth inhibition at a target site [5]. The derivative, named eMIP (or ECI301), is a single amino acid-substituted, 69-amino acid derivative of CCL3. Marked infiltration of $CD4^+$ and $CD8^+$ cells, respectively, were observed not only in the tumor at the irradiated site but also at the non-irradiated site [5]. Depletion of any of NK1.1, $CD4^+$ and $CD8^+$ cells diminished the abscopal effect by eMIP [5]. On the other hand, splenocytes could secret IFNγ upon exposure to the same type of tumor cells [6]. This provides evidence that these lymphocytes are involved in both the enhanced tumor type-specific and non-specific tumor cell killing.

We further found that intratumoral injection of tumor cell lysates followed by intravenous administration of eMIP can inhibit tumor growth, not only at the site of injection, but also at non-treated sites. These effects of the tumor lysate were recapitulated by intratumoral injection of alarmin HSP70, while administration of a neutralizing HSP70 antibody neutralized the cooperative effects of eMIP on tumor irradiation [6]. Furthermore, intravenous administration of HSP70 + eMIP was sufficient to inhibit tumor growth, though not completely [6]. These studies revealed mechanistic insights into the basis for the abscopal effect of radiotherapy. Tumor-derived extracellular Hsp70 has been recognized as a

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chaperokine (molecular chaperon with cytokine activity), activating antitumor immunity [7].

To distinguish the active portion of eMIP, partial peptides of eMIP were chemically synthesized and their activities were examined. In this paper, we show that intravenous administration of a 30-amino acid partial peptide of eMIP induces the abscopal effect after local irradiation of a target tumor. In contrast to eMIP and CCL3, the partial peptide has no chemotaxis-inducing activity.

Materials and methods

Materials

Recombinant eMIP, a 69-amino acid variant of human CCL3 (Fig. 1) carrying a single amino acid substitution of Asp27 to Ala, was generated using an *S. pombe* (Asahi Glass Co. Ltd.) or *Corynebacterium glutamicum* expression system (Ajinomoto Co. Ltd) and purified to homogeneity [5]. A structural map of eMIP is shown in Fig. 1C. It contains two alpha-helix and one β -sheet. "N" and "C" indicate the N- and C-termini respectively. Three partial peptides (Fig. 1D–F) were chemically synthesized by solid-phase peptide synthesis (Eurofins Genomics, Tokyo). Recombinant mouse HSP70-A1 (low endotoxin) was obtained from Enzo Biochem. Mouse monoclonal anti-human CCL3 (R&D) and anti-HSP70 antibodies (Stress Marq), and biotin labeled, rabbit polyclonal anti-CCL3 antibody (R&D) were used for immunoprecipitation analysis.

Mouse

Six-week-old male BALB/c mice were purchased from Nippon SLC (Hamamatsu, Japan) and housed in a barrier system with controlled light (12L: 12D) and temperature (22 ± 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.

Tumor cells

Colon26 adenocarcinoma cells were provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan), as described previously [6]. The cultures were maintained at 37 °C under humidified 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal bovine serum, for no longer than 12 weeks after recovery from frozen stocks. Before tumor implantation, cells were trypsinized and filtered through a 70 μm pore size cell strainer. Colon26 cells were inoculated subcutaneously in the left and right flanks of BALB/c mice. When the mass of solid tumor at the right flank reached 8–10 or 5–8 mm diameter (14–18 days after implantation), mice with similar-sized tumors were selected from more than 100 tumor-injected mice and used for each experiment. Number of mice used in a group were 7 or 8 (n = 7. n = 8). Only the right side was exposed to radiation (6 Gy).

The following formula was used for calculation of tumor volume.

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

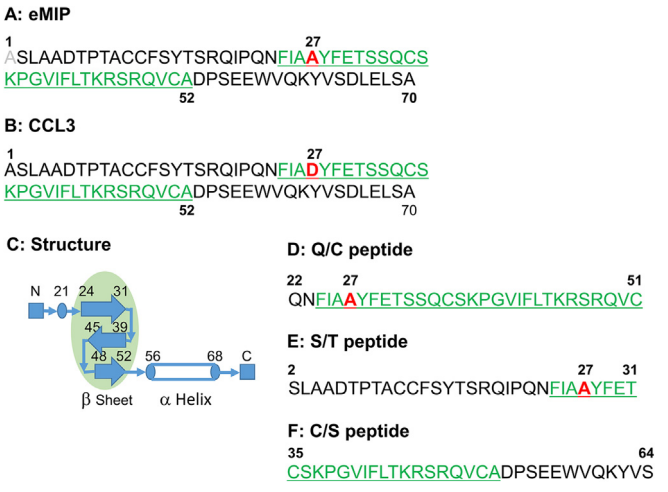

Fig. 1. Amino acid sequences of eMIP (A), CCL3 (B) and synthetic peptides (D–F), and the structure of eMIP /CCL3. Amino acid numbers are based on those of CCL3. Ala1 of CCL3 is missing in eMIP, and Asp27 of CCL3 is substituted to Ala in eMIP (Red). In a model structure of eMIP (C), β-Sheet region is exhibited in a green oval. An N-terminal loop contributes to dimer formation between two single molecules. Underlined amino acids (Green) and those in the green disc (Structure) form a β sheet. Sequences of Q/C (D), S/T (E) and C/S (F) peptides are shown at right.

Tumor irradiation

Mice bearing tumors were held in the decubitus position using mouse irradiation fixture, and ionizing radiation (6 MeV-electron beam) was delivered to the right flank from just above the tumor [5]. Electron beam was chosen to treat the subcutaneously injected, superficial tumor. 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 absorbed dose into the dorsal spine was calculated to be below 10%. Optimum radiation dose was determined to be 6 Gy whereas single 2 or 10 Gy doses were too weak or too strong, respectively, to show the effect of eMIP.

Molecular mass determination of eMIP and CCL3

The Agilent 1200 series high performance liquid chromatography system (HPLC) was used for molecular mass determination employing the TSKgel G2000SWXL 6 x 250 mm column (Tosoh Bioscience). Flow rate was adjusted to 0.5 ml/ml. UV 215 was used for sample detection. Reference samples of molecular mass standards were obtained from BioRad and Sigma.

Chemotaxis assay of eMIP, CCL3 and synthetic peptides

For chemotaxis analyses, we used either CCR1-expressing Jurkat cells or THP-1 cells depending on the methods employed An optically accessible horizontal chemotaxis apparatus, TAXIScan [8,9], with a 5-μm deep microchannel chip was used for chemotaxis assay of CCR1-expressing Jurkat cells toward eMIP and CCL3. Migration velocity was calculated by TAXIScan analyzer 2 [10]. Using a 48-well Micro Chemotactic Chamber (Neuro Probe, Gaithersburg, MD), migration of THP1 cells added to the upper wells (1×10^5 cells/50 μl/well) was evaluated by adding each synthetic peptide or eMIP to the lower wells (25 μl). Data are expressed as the migration index, the relative number of cells migrated toward each peptide or eMIP against the number of cells migrated toward the control without attractant.

Statistical analysis

Data are presented as mean ± SE, unless otherwise stated. We used ANOVA for comparisons between groups in the *in vivo* study. Significance of the difference between the means of two variables was determined by the Dunnett's test unless otherwise stated. A P value <0.05 was considered significant.

Results

Comparison of abscopal eliciting activity between eMIP and CCL3

Although eMIP is a 69-amino acid variant of human CCL3 carrying only a single amino acid substitution of Asp27 to Ala, we found that intravenous administration of eMIP induced the abscopal effect after local radiation treatment, while corresponding treatment with CCL3 did not. In a model experiment using Colon26 cells inoculated in the left and right flanks of BALB/c mice (Fig. 2A), a single dose of 6 Gy was delivered to the right-side tumor 16 days after tumor implantation. Starting from the next day, 2 or 10 μg CCL3 or 2 μg control eMIP were administered intravenously once per day for 5 consecutive days. Tumor growth both at the target and non-targeted sites was markedly inhibited by irradiation + eMIP (Fig. 2A). We have shown previously [5,6] that this combination treatment also elicits the abscopal effect with other syngeneic tumor grafts, including MethA fibrosarcoma (in BALB/c mice), Lewis lung carcinoma (in C57BL/6 mice) and FM3A carcinoma (C3H/HeN). In contrast to eMIP, CCL3 did not inhibit tumor growth at the non-irradiated site (Fig. 2A).

Although the basic structure of one molecule of both eMIP and CCL3 is known to be a dimer [11,12], CCL3 used in the present study forms a double helical polymer (Fig. 2B), burring its receptor-binding sites, whereas eMIP forms only tetramers and dimers. Compared with eMIP, CCL3 does not bind to HSP70 effi-

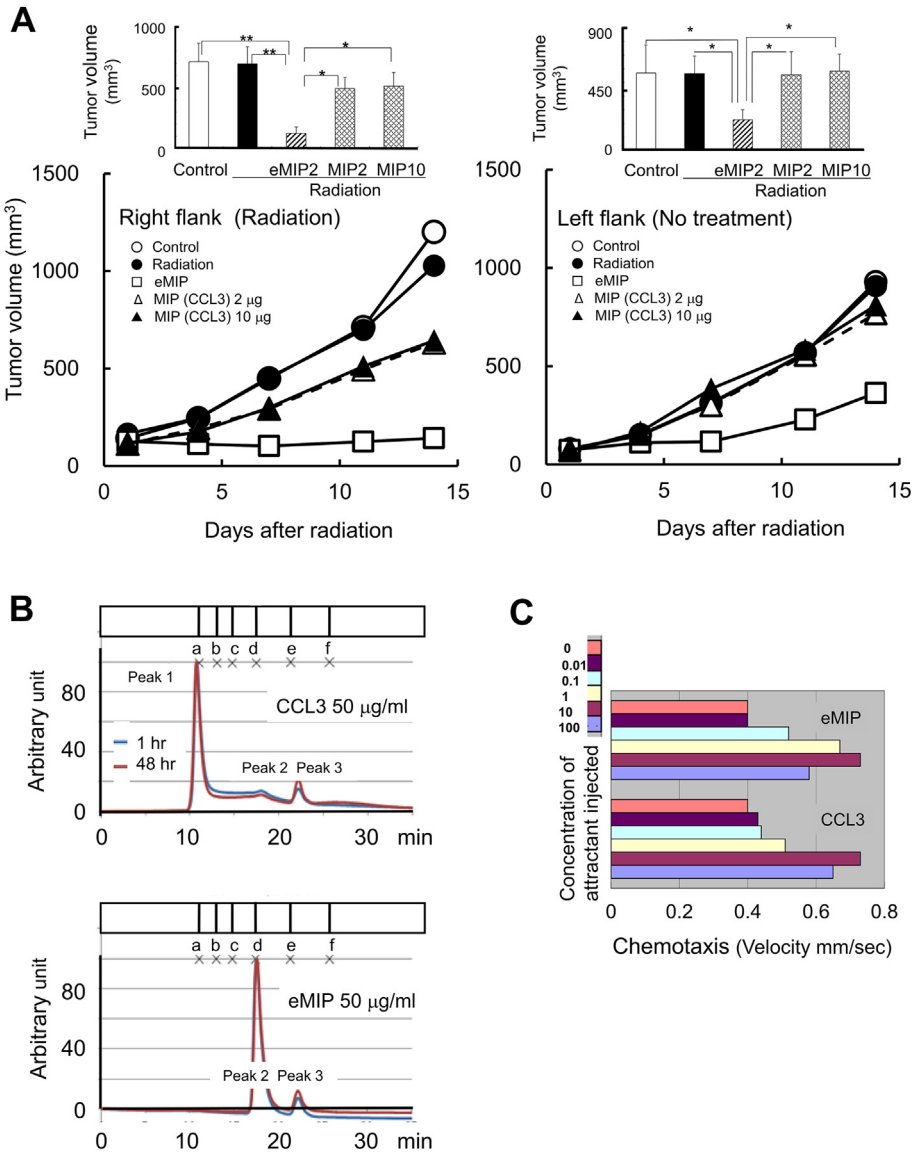

Fig. 2. Activities of eMIP and CCL3, and their structure. (A) Effect of eMIP or CCL3 (indicates as MIP in the figure) on tumor growth administered after local radiation treatment. Time course of tumor growth at irradiated (left) and non-irradiated sites (right), and tumor volume at 11 days after irradiation (inset) are shown. Colon26 cells were inoculated in the left and right flanks of BALB/c mice; 16 days after inoculation only the right side was exposed to radiation (6 Gy). MIP (CCL3: 2 or 10 μg/0.2 ml/mouse) or eMIP (2 μg/0.2 ml/mouse) was given 5 times daily starting 20 h after irradiation. Mice with radiation treatment only or without treatment served as controls. Each bar indicates mean volume ± SE (n = 7). Each symbol represents mean tumor volume of control (○), radiation treatment (●), 2 μg eMIP (□), 2 (Δ) or 10 μg MIP (CCL3) (▲) given after irradiation. Significant differences: $^{*}P < 0.05$; $^{**}P < 0.01$ (ANOVA). (B) Molecular masses of CCL3 and eMIP analyzed by HPLC. The thawed frozen-stock samples were subjected to HPLC after incubation at 4 °C for 1 or 48 h. The main peak of CCL3 was a 60mer (Peak 1) and second peak was a dimer (Peak 3). eMIP was a mixture of tetramer (Peak 2) and dimer (Peak 3). Lines in the box above each chart show peak positions of standard proteins: a-f, in this order, thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa) and aprotinin (6.5 kDa). (C) Migration of CCR1-expressing Jurkat cells towards a concentration gradient of eMIP or CCL3 analyzed using TAXIScan technology. After aligning the cells along the start line on the edge of the channel, the indicated concentration (nM) of eMIP or CCL3 was applied to the other compartment. Time-lapse images were taken every 30 s for 60 min and mean velocity of cell migration was calculated.

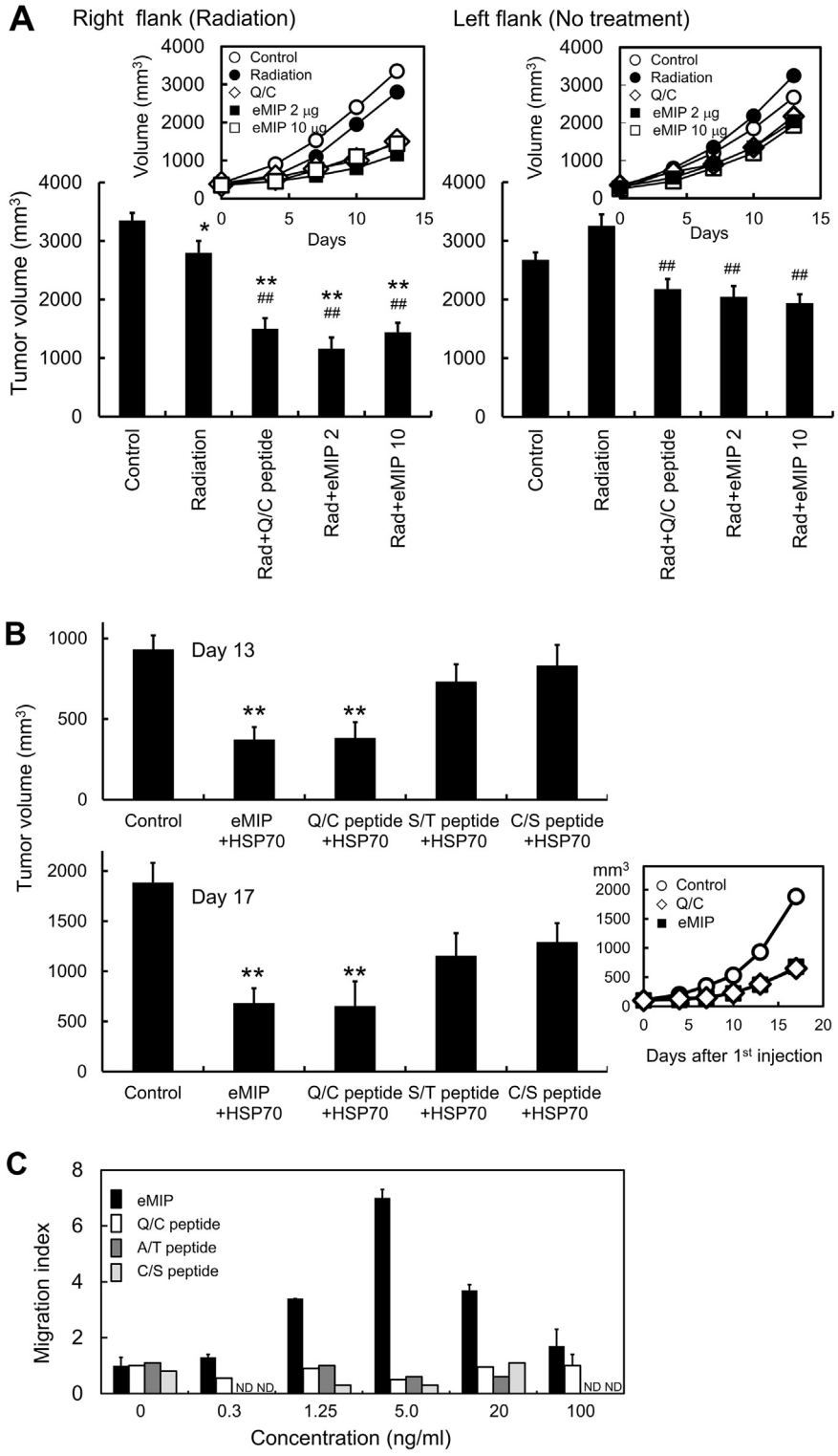

Fig. 3. Activities of synthetic peptides. (A) Effect of Q/C peptide on tumor growth at irradiated and non-irradiated sites, administered after local radiation treatment. Colon26 cells were inoculated in the left and right flanks of BALB/c mice. A single dose of 6 Gy was delivered to the right-side tumor 14 days after tumor implantation. Starting next day, 10 μg eMIP equivalent dose of Q/C peptide, or 2 or 10 μg eMIP was administered intravenously once a day for 5 consecutive days. Mean tumor volumes at 13 days after radiation treatment are shown. Each bar indicates mean volume ± SE (n = 8). Significant differences from the control are indicated by: $^{*}p < 0.05$ or $^{**}p < 0.01$, and from the radiation group by: $^{\#\#}p < 0.01$ (ANOVA). Inset: Time course of tumor growth after irradiation, where the horizontal axis indicates days after radiation treatment. Each symbol represents mean tumor volume of control (○), radiation treatment (●), Q/C peptide (◇), 2 (■) or 10 μg eMIP administrated (□) after radiation. (B) Effect of i.v.-administered mixtures of partial peptides of eMIP with HSP70 on tumor growth. Colon26 cells were implanted in the right flank of BALB/c mice. When the tumor size reached 5–8 mm diameter (14 days), animals received mixtures of 0.5 μg HSP70 and 10 μg eMIP-equivalent dose of one of Q/C, S/T, C/S peptides or 2 μg eMIP in a total volume of 0.2 ml PBS, intravenously 3 times on days 0, 1 and 2. Each bar indicates mean volume ± SE (n = 8) at day 13 and 17. Significant differences from the control and radiation groups are indicated by: $^{**}p < 0.01$ (ANOVA). Inset: time course of tumor growth. Each symbol represents mean tumor volume of control (○), Q/C peptide (◇) and eMIP (■). (C) Chemotaxis-inducing activity of synthetic peptides and eMIP. Migration index shows the relative number of migrated cells toward each peptide, or eMIP, versus number of cells migrated toward buffer without attractant.

ciently when assayed by immunoprecipitation using anti-HSP70 coupled beads. Hence, efficient complex formation with HSP70 seems to be critical for exhibiting the antitumor activity of eMIP. It was shown that purified HSP70 cannot stimulate the immune system unless by binding other molecules [13]. In this context, we have previously shown that intravenous administration of the combination of HSP70 + CCL3 was unable to mimic the combination treatment of radiation with eMIP, in contrast to the combination of HSP70 + eMIP [6].

Antitumor activity of the partial peptides of eMIP administrated after irradiation

Originally, we thought that the chemotaxis inducing ability of eMIP (Fig. 2C) could play an important role in its antitumor activity. However, since CCL3 did not elicit the abscopal effect, chemotaxis-inducing activity is unlikely to relate directly to the anti-tumor activity, but rather seems to work only when combined with another molecule like HSP70, as shown above. We, therefore, sought a partial peptide of eMIP with a similar abscopal effect inducing activity as eMIP but provably without chemotaxis inducing activity. We made three chemically-synthesized partial peptides of eMIP, each consisting of 30 partially overlapping amino acids (Fig. 1D–F), two of which include the Asp27 to Ala amino acid substitution (Bold).

To evaluate the abscopal effect inducing activity of these peptides, similar experiments as those shown in Fig. 2A were carried out using each peptide instead of eMIP. We found that one of the peptides, Q/C, containing the substituted amino acid, had almost equivalent activity to eMIP. Fig. 3A shows tumor volumes of Colon 26 cells in left and right flanks 13 days after radiation treatment, and the inset shows the time course of tumor growth. Tumor growth at the radiation treated right flank was slightly inhibited, and the tumor volume was smaller than that of control animals ($P < 0.05$). After administration of either eMIP or Q/C peptide, a significant and similar level of decrease in tumor volume was observed both in radiation-treated and non-treated sites, as compared to the radiation-only group ($P < 0.01$). These results indicate that the homologous sequence of Q/C peptide derived from eMIP plays an important role in the enhanced tumor growth inhibition of this chemokine.

Tumor growth inhibition by the partial peptides co-administered with HSP 70

We found previously that intravenous administration of HSP70 + eMIP inhibited tumor growth [6]. Here, we tested if intravenous administration of the HSP70 + partial-peptide combination could mimic the combination treatment of radiation with eMIP. Colon 26 cells subcutaneously implanted in the right flank of BALB/c mice were allowed to grow for 14 days until the tumor size reached 5–8 mm diameter. Mixtures of HSP70 and one of either Q/C, S/T or C/S peptide, or eMIP, were intravenously administered once per day for 3 consecutive days. As shown in Fig. 3B, clear tumor growth inhibition was observed after the administration of either eMIP or the Q/C peptide in combination with HSP70 ($p < 0.01$). Like the combination treatment with radiation, tumor growth inhibition by eMIP + HSP70 and Q/C peptide + HSP70 was observed from a very early stage of treatment. Other peptides showed a tendency to inhibit tumor growth, though the levels were not statistically significant.

Chemotaxis-inducing activity of the partial peptides and eMIP

To confirm that these peptides have no chemotaxis-inducing ability, we performed a chemotaxis assay using THP-1 cells, which

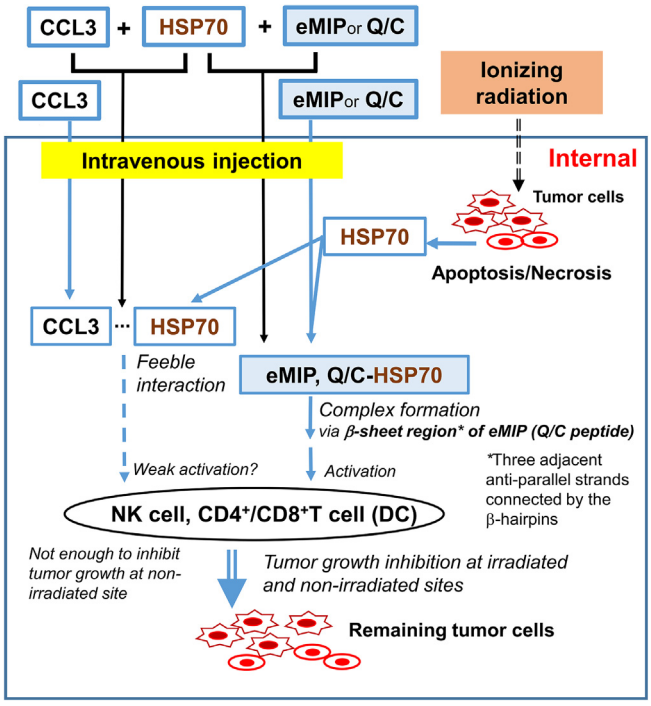

Fig. 4. Possible action mechanism of eMIP, CCL3 and Q/C peptide. Local irradiation induces apoptosis and necrosis of rapidly dividing tumor cells, from which HSP70 is released. i.v.-Administrated eMIP and probably Q/C peptide also forms a complex with HSP70, which in turn promotes eradication of tumor cells that may evade the treatments by stimulating NK cells and $CD4^+$ and $CD8^+$ T cells, directly or indirectly. Activated DC by the complex may concern activation of these cells. i.v.-Injected mixtures of HSP70 + eMIP and HSP70 + Q/C peptide also inhibited tumor growth. The lower binding activity of CCL3 to HSP70 makes its biological activities weaker than eMIP and, as a result, it cannot induce the abscopal effect after radiation treatment and anti-tumor activity by administration with HSP70. Further studies are needed to elucidate the precise role of eMIP/HSP70 or Q/C peptide/HSP70 in recruitment and activation of $CD4^+$, $CD8^+$ and NK cells at the tumor bearing sites.

are known to express the receptor for CCL3. As shown in Fig. 4, THP-1 cells did not show any chemotactic response to the Q/C or other peptides at concentrations from 0.3 to 200 ng/ml, whereas the same cells showed a typical bell-shaped response curve to eMIP with a peak at 5 ng/ml. These results indicate that chemotaxis-inducing activity and enhanced tumor growth inhibition are independent eMIP phenomena.

Discussion

In this paper, a 30-amino acid eMIP peptide—Q/C peptide, named for the amino acids at the N- and C-termini-was shown to inhibit tumor growth not only following local irradiation but also by co-administration with HSP70. This activity was comparable to that seen when eMIP was used instead. Although the experimental setup used in this analysis was not appropriate to examine the presence of an abscopal effect, we have previously shown that eMIP did induce a better tumor control in the contralateral unirradiated tumor in the combination group compared to eMIP systemic treatment alone [5].

Fig. 4 summarizes the results and possible mechanism of action for these molecules based on the collective findings to date [5,6]. Although we did not include role of HMGB1 in this figure, HMGB1 released from tumor cells seems to work subsequently to HSP70 [6,14]. The sequence of the Q/C peptide (Fig. 2D) corresponds to the β-sheet region of eMIP (Fig. 2C), consisting of 3 adjacent anti-parallel strands (24Phe-31Thr, 39Gly-45Lys and 48Arg-52Ala) connected by the β-hairpins (PDBsum entry, cord 2 × 69, Bioinformatics Services, EMBL-EBI website). At least two β-strands in the Q/C

peptide can be predicted based on secondary structure prediction by Chou-Fasman, GOR and neural network (ver. 1.1) [15–17]. Therefore, it is possible that these secondary structures of eMIP, and hence its conformation, is important to activate immune cells when co-administered with HSP70. The chemotaxis-inducing ability of eMIP appears unrelated to its anti-tumor activity. Although parts of the other peptides overlap and contain at least one potential β strand, they showed statistically non-significant inhibition of tumor growth.

Due to the various limitations of existing antitumor therapies, it should benefit patients to develop combination therapies. In the case of radiation therapy, we have shown with a model experiment that intravenously administered eMIP enhanced the antitumor effect following irradiation, and induced the tumor-independent and -dependent abscopal effect [5] and, as shown in this paper, the effect of eMIP can be recapitulated with the Q/C peptide. For clinical trials, chemically synthesized peptide like Q/C peptide has various advantages. Compared to recombinant proteins like eMIP, production and quality control of peptides are much easier and production cost is very low. Combination therapies recently reported using cytokines/chemokines was the combination of radiotherapy with granulocyte-macrophage colony-stimulating factor (GMCSF), a potent simulator of dendritic cell (DC) maturation. This therapy produced abscopal responses in some patients with metastatic solid tumors [18], although GMCSF is known to be highly immunogenic in some patients and causes an autoimmune disease [19]. In the case of eMIP and Q/C peptide, Toll like receptor 4 (TLR4)-expressing DC's are considered to be activated by these molecules in complex with HSP70, which in turn activates helper and killer T cells. We have shown previously that mice genetically deficient in TLR4, an immune cell receptor that binds HSP70, did not exhibit antitumor responses to irradiation + eMIP [6].

It was suggested that a raised neutrophil to lymphocyte ratio in irradiated patients could be a marker for poor prognosis in GMCSF treatment [18]. The meta-analysis of 100 studies comprising 40,559 patients revealed that prognosis was poor in the patients with a neutrophil to lymphocyte ratio greater than 4 [20]. In the case of local radiation treatment, the reduced number of lymphocytes seems to be responsible for raised neutrophil/lymphocyte ratios and patient prognosis, since in our own Phase I clinical studies for the combination of radiotherapy with eMIP, a half of patients examined showed reduced number of lymphocytes. Lymphocytes are highly sensitive to irradiation and, in contrast to neutrophils, hardly recover once depleted. To enhance host defense mechanism most efficiently, development of a treatment to keep lymphocytes intact seems to be required.

Conclusion

The present data provide evidence for an understanding of the underlying mechanism for the abscopal effect induced by irradiation + eMIP, though further studies are necessary to elucidate the more precise role of eMIP and Q/C peptide in their anti-tumor activity. Such data will encourage future therapeutic application of like peptides in the treatment of advanced metastatic cancer.

Conflict of interest statement

The authors declare no potential conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ctro.2018.02.004.

References

[1] Friedman EJ. Immune modulation by ionizing radiation and its implication for cancer immunotherapy. Curr Pharm Des 2002;8:1765–80.
[2] Garnett CT, Palena C, Chakarborty M, Tsang K, Schlom J, Hodge JM. Sublethal irradiation of human tumor cells modulates phenotype resulting in enhanced killing by cytotoxic T lymphocytes. Cancer Res 2004;64:7985–94.
[3] Sologuren I, Rodríguez-Gallego C, Lara PC. Immune effects of high dose radiation treatment: implications of ionizing radiation on the development of bystander and abscopal effects. Transl Cancer Res 2014;3:18–31.
[4] Rödel F, Frey B, Multhoff G, et al. Contribution of the immune system to bystander and non-targeted effects of ionizing radiation. Cancer Lett 2015;356:105–13.
[5] Shiraishi K, Ishiwata Y, Nakagawa K, et al. Enhancement of antitumor radiation efficacy and consistent induction of the abscopal effect in mice by ECI301, an active variant of macrophage inflammatory protein-1α. Clin Cancer Res 2008;14:1159–66.
[6] Kanegasaki S, Matsushima K, Shiraishi K, et al. Macrophage inflammatory protein derivative ECI301 enhances the alarmin-associated abscopal benefits of tumor radiotherapy. Cancer Res 2014;74:5070–8.
[7] Juhasz K, Lipp AM, Nimmervoll B, et al. The complex function of HSP70 in metastatic cancer. Cancer 2014;6:42–66.
[8] Kanegasaki S, Nomura Y, Nitta N, et al. A novel optical assay system for the quantitative measurement of chemotaxis. J Immunol Methods 2003;282:1–11.
[9] Nitta N, Tsuchiya T, Yamauchi A, et al. Quantitative analysis of eosinophil chemotaxis tracked using a novel optical device-TAXIScan. J Immunol Methods 2007;320:155–63.
[10] Yamauchi A, Degawa-Yamauchi M, et al. Systematic single cell analysis of migration and morphological changes of human neutrophils over stimulus concentration gradients. J Immunol Methods 2014;404:59–70.
[11] Graham GJ, MacKenzie J, Lowe S, et al. Aggregation of the chemokine MIP-lα is a dynamic and reversible phenomenon. Biochemical and biophysical analysis. J Biol Chem 1994;269:4974–8.
[12] Ren M, Guo Q, Guo L, et al. Polymerization of MIP-1 chemokine (CCL3 and CCL4) and clearance of MIP-1 by insulin-degrading enzyme. EMBO J 2010;29 (23):3952–66.
[13] Tsan MF. Heat shock proteins and high mobility group box 1 protein lack cytokine function. J Leukoc Biol 2011;89:847–53.
[14] Kanegasaki S, Tsuchiya T. Alarmins released during local antitumor treatments play an essential role in enhancing tumor growth inhibition at treated and non-treated sites via a derivative of CCL3. OncoImmunology 2014;3(10): e958956.
[15] Prevelige Jr P, Fasman GD. Prediction of the secondary structure of proteins: the Chou-Fasman-Prevelige algorithm. In: Fasman GD, editor. Prediction of protein structure and the principles of protein conformation. New York: Plenum; 1989. p. 391–416.
[16] Garnier J, Osguthorpe DJ, Robson B. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J Mol Biol 1978;120:97–120.
[17] Qian N, Sejnowski TJ. Predicting the secondary structure of globular proteins using neural network models. J Mol Biol 1988;202:865–84.
[18] Golden EB, Chhabra A, Chachoua A, et al. Local radiotherapy and granulocyte-macrophage colony-stimulating factor to generate abscopal responses in patients with metastatic solid tumours: a proof-of-principle trial. Lancet Oncol 2015;16:795–803.
[19] Kitamura T, Uchida K, Tanaka N, et al. Serological diagnosis of idopathic pulmonary alveolar proteinosis. Am J Respir Crit Care Med 2000;162:658–62.
[20] Templeton AJ, McNamara MG, Šeruga B, et al. Prognostic role of neutrophil-to-lymphocyte ratio in solid tumors: a systematic review and meta-analysis. J Natl Cancer Inst 2014;106:dju124. https://doi.org/10.1093/jnci/dju124.