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Combination of mutated herpes simplex virus type 1 (G207 virus) with radiation for the treatment of squamous cell carcinoma of the head and neck

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Abstract

G207 is an oncolytic herpes simplex virus (HSV) with deletions at both $\gamma_1$34.5 loci and a *LacZ* gene insertion inactivating the HSV ribonucleotide reductase gene. Ionising radiation induces the growth arrest-inducible gene, *GADD34*, and ribonucleotide reductase. GADD34 is a protein that correlates with apoptosis following radiation and has homology with the G207 $\gamma_1$34.5 gene. We hypothesised that the combination of radiotherapy with G207 may have a potentiating effect on viral replication and anti-tumour efficacy. The purpose of this study was therefore to evaluate the combination of G207 with radiation therapy to treat head and neck tumours. The cytotoxicity of G207 was tested in six head and neck squamous carcinoma cell lines, in the presence or absence of irradiation. For *in vivo* experiments, flank tumours in C3H/HeJ mice or in nude mice were treated with direct injections of G207, with or without radiation. All head and neck squamous cancer cell lines tested demonstrated significantly increased antitumour effects with the combination of G207 virus and radiation therapy compared with each individual modality ($P < 0.01$). Furthermore, the combination treatment effect was better than the expected additive effect of the two therapies in combination. Even the radiation-resistant cell lines (SCC25, MSKQLL2, SCCVII) were susceptible. The combination of direct G207 injection with radiation therapy suppressed human and murine squamous cell carcinoma growth significantly ($P < 0.05$ and $P < 0.001$) compared with controls or single modality therapy. G207 enhanced the effectiveness of radiation therapy and low-dose radiation potentiated the effectiveness of G207 viral therapy in head and neck cancer. These findings suggest a potential clinical application for this combined therapy as treatment for radiation-resistant head and neck cancers.

Keywords: Gene therapy; Herpes simplex virus; Radiation; Head and neck neoplasm

1. Introduction

Head and neck cancer composes 6.6% of all new cases of malignant disease in the US [1]. One-half of these patients present with advanced stage disease, and only approximately one-third of these patients survive 5 years with presently available therapies [2]. This cohort of patients may benefit from the development of novel therapies to improve locoregional control and survival. In recent years, gene therapy has been evaluated as a potential treatment for squamous cell carcinomas of the head and neck [3–5]. Oncolytic viral therapy is an approach that uses genetically engineered virus to directly kill tumour cells. The ideal viral candidate would

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be oncolytic in a wide variety of tumours, replication-competent, safe and susceptible to antiviral therapies.

G207 virus is a mutated herpes simplex virus (HSV) type 1 with deletions at both $\gamma_1$34.5 loci and a *lacZ* insertion interrupting the ICP6 gene that encodes the HSV ribonucleotide reductase. The abrogation of the HSV ribonucleotide reductase enables G207 to replicate more selectively in rapidly dividing cells with higher endogenous ribonucleotide reductase levels. Viral replication is highly attenuated in non-dividing cells, permitting a more targeted infection and lysis of malignant cells. G207 has also been attenuated by deletions of both $\gamma_1$34.5 loci, which results in significantly decreased neurovirulence. Other qualities of G207 that may enhance its safety for clinical use are sensitivities to high temperatures, impeding viral replication in the presence of fever, and anti-herpetic drugs, such as acyclovir or gancyclovir. In addition, the multiple large mutations in G207 make spontaneous reversion to a wild-type HSV phenotype extremely unlikely. G207 also expresses the *lacZ* gene to serve as an easily identifiable marker of infection. These many favourable traits make G207 an attractive candidate for use in oncolytic viral therapy [6,7].

There are a number of reasons to suspect that oncolytic viral therapy may be combined with radiation therapy and result in increased anti-tumour efficacy. Ionising radiation induces the expression of the growth arrest-inducible gene, *GADD34*. GADD34 has a high degree of homology with the G207 $\gamma_1$34.5 [8,9], and may have a functional homology to $\gamma_1$34.5 that potentially enhances G207 activity in radiated tumour tissues. In addition, ionising radiation induces a transient increase in ribonucleotide reductase activity [10] and may also enhance tumour killing. The mechanisms of cytotoxicities are different between oncolytic viral therapy and radiation therapy and could theoretically target tumour killing with their combined application.

We hypothesised that the combination of radiation and G207 viral therapy may have a synergistic effect on viral replication and cytotoxicity. The purpose of this study was to evaluate the use the oncolytic G207 virus in combination with radiation therapy to treat head and neck cancer. Additive or synergistic effects detected with combination therapy would support the potential clinical application of this strategy in treating patients with head and neck cancer.

2. Materials and methods

2.1. Cell lines

Five human head and neck squamous cell carcinoma lines and one murine squamous cell carcinoma line were used in this study. Three lines (SCC 1483, MSK QLL2, and HN886) were isolated and characterised at Memorial Sloan-Kettering Cancer Center, and two (SCC 15 and SCC 25) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The SCCVII murine cell line is a cutaneous squamous cell cancer that spontaneously arose in the C3H/HeJ mouse. For *in vitro* studies, cells were grown in minimal essential media supplemented with 10% foetal calf serum (FCS) at 37 °C in 5% CO_2 humidified atmosphere and subcultured once or twice a week.

2.2. Animals

Male C3H/HeJ mice (Jackson Laboratory, Bar Harbor, ME, USA) at least 8 weeks of age were maintained in pathogen-free conditions and were used for experiments with SCCVII. A murine model of oral cavity cancer has previously been described using the SCCVII cell line [11]. For xenograft head and neck cancer model, six-week-old athymic nude mice (National Cancer Institute, Bethesda, MD, USA) were maintained in a pathogen-free environment within the animal facility. All experimental protocols were reviewed and approved by the Institutional Animal Care Committee of Memorial Sloan-Kettering Cancer Center.

2.3. G207 Virus

The G207 virus was constructed as previously described with deletions of both copies of the $\gamma_1$34.5 gene and a *lacZ* gene insert into the ICP6 locus [7]. The G207 virus was propagated in African green monkey kidney cells (Vero Cells, ATCC, Manassas, VA), which were maintained in Dulbecco's modified Eagle's medium (DMEM). Vero cells were infected with the G207 virus at an multiplicity of infection (MOI) of 0.02 at 34 °C. The G207 virus was harvested after 2 days and subjected to freeze–thaw lysis to release the intracellular virus. Cell lysates were centrifuged (300 g for 10 min at 4 °C) and viral supernatants were aliquoted and stored at −80 °C. Viral titres were determined on Vero cells by plaque assay.

2.4. In vitro cytotoxicity assay

Six head and neck squamous cancer cell lines were harvested from exponential-phase maintenance cultures and a single-cell suspension was plated at a density of 1×10^4 cells/200 μl media into 96-well, flat-bottomed culture plates. After incubation at 37 °C for 24 h, the plates were divided into four groups. The control and G207 alone groups received no irradiation. The radiation alone and combination of G207 with radiation groups were treated with 400 cGy using a ^{137}Cs source irradiator (Mark I Irradiator; Sepherd and Associates, CA). This dose of radiation was chosen after prelimi-

nary experiments demonstrated this to be a dose that produced an intermediate level of cell killing. The G207 groups were then infected with G207 virus at a MOI of 0.1 (MOI of 1 in SCCVII) by adding the appropriate concentration of virus in 200 μl of media. All assays were performed in quadruplicate, and control wells were treated with medium alone. Plates were assayed daily for 5 days by aspirating the culture medium, washing with phosphate-buffered solution (PBS), and adding 200 μl of media containing 50 μg of 3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). After 4 h of incubation, the MTT media was slowly aspirated from each well and replaced with 200 μl dimethyl sulphoxide (DMSO). The absorbance of each well was measured using a micro-culture plate reader (EL312e, Bio-Tek Instruments, USA) at 540 nM.

2.5. In vitro viral proliferation assay

Viral replication of G207 in non-radiated and radiated cells was compared in six different head and neck squamous cancer cell lines (SCC1483, SCC15, SCC25, MSKQLL2, HN886, SCCVII) using the standard plaque assay [12]. During the MTT cytotoxicity experiment described above, culture media from both non-radiated and radiated cells was collected daily for 5 days after infection with G207 at an MOI of 0.1 (MOI of 1 in SCCVII). All media samples were centrifuged at 2000 rotations per minute (rpm) for 10 min at 4 °C. Supernatants were then plated on confluent Vero cells in 6-well plates at varying dilutions, and viral titres were determined using the standard plaque assay [12].

2.6. In vitro assay of the infection efficacy

Each of the six head and neck squamous cancer cell lines was plated into 12-well plates at 1×10^5 cells per well in 0.9 ml of media. Twenty-four hours later, one group of cells was not radiated, while the other group was radiated to 400 cGy with a ^{137}Cs source irradiator (Mark I Irradiator; Sepherd and Associates, CA). Then, the cells were exposed to G207 at a MOI of 0.1 by adding 100 ml of virus in media to each well. After incubation for 24 h, cells were stained with X-gal (5-bromo-4-chloro-3-indol-β-D-galactopyranoside) as previously described and the percent of blue-staining cells calculated [13].

2.7. In vivo inhibition of tumour growth

2.7.1. SCCVII syngeneic mouse model

C3H/HeJ mice were randomly divided into four groups. In each group, subcutaneous (s.c.) SCCVII flank tumours were established by injecting 1×10^6 cells in 50 μl PBS. Animals were monitored on a daily basis for tumour growth until the implanted tumours reached 0.5 cm in maximum diameter over approximately 5 days. When most of the animals had 0.5 cm diameter tumour nodules, all of the animals in each group were normalised according to tumour size and animal weight ($n = 10$ per group). Animal groups were treated as follows: *group I* (control): intratumoral injections of PBS; *group II*: intratumoral injections of 1×10^7 plague-forming units (pfu) G207; *group III*: 500 cGy radiation and intratumoral injections of PBS; *group IV*: intratumoral injections of 1×10^7 pfu G207 and 500 cGy radiation. Tumours treated with G207 were injected for a total of two doses two days apart. The animals were examined daily and their weights and tumour volumes were recorded [14].

2.7.2. Human SCC xenograft in athymic nude mice model

Another *in vivo* study was done to rule out the immune effect in the combination treatment and evaluate the treatment effect in human head and neck squamous cancer xenograft model. Four to six week old athymic nude mice were maintained in a pathogen-free environment within the animal facility. The mice were anaesthetised intraperitoneally (i.p.) with pentobarbital (50 mg/kg) and 2×10^6 SCC15 tumour cells in 50 μl PBS were injected s.c. into both flanks. When the tumours had reached approximately 5 mm in diameter, one of the following preparations was treated: *group I* (control): PBS alone; *group II*: 1×10^7 pfu G207 virus; *group III*: radiation and intratumoral injections of PBS; *group IV*: combination of G207 virus injection (1×10^7 pfu G207 virus) with radiation. Radiation dose was 500 cGy. Immediately after irradiation, a single dose of G207 virus was injected intratumorally. The animals were examined daily and their weights and tumour volumes were recorded as described above [14].

2.8. In vivo viral proliferation assay

In C3H/HeJ mice, s.c. bilateral flank tumours were established as described above. When the tumours reached approximately 7–9 mm in diameter, half of the animals received irradiation at 500 cGy. Animal groups were as follows ($n = 15$ per group): *group I* (control): intratumoral injections of G207; *group II*: 500 cGy radiation and intratumoral injections of G207. All animals were then treated with a single intratumoral injection G207 (1×10^6 pfu). Over 5 days, three animals from the each group were sacrificed and tumours were excised daily. Tumours were frozen in 1 cc media, thawed, homogenised, and subjected to three freeze–thaw cycles. Samples were then vortexed and centrifuged at 1500 rpm × 5 min. The viral titres of supernatants were determined by the plaque assay at varying dilutions as described above [12].

2.9. Statistical analysis

All data are expressed as mean ± standard deviation of the mean. Comparisons between groups were made using the two-tailed Student's *t*-test. Analysis of variance (ANOVA) or repeated measured ANOVA and Tukey multiple comparison tests were used, where appropriate, to identify statistical significance for multiple comparisons.

3. Results

3.1. In vitro cytotoxicity assay

All of the head and neck squamous carcinoma cell lines tested demonstrated an increased susceptibility to treatment with the combination of G207 and radiation therapy compared with control or single modality therapy. Five days after irradiation of the SCC1483 cell line with 400 cGy, approximately 23% of the tumour cells were still viable. Approximately 24% of the tumour cells were viable after administration of the G207 virus alone. However, all tumour cells were eradicated with a combination of radiation and G207 virus therapy. The effectiveness of combination treatment was confirmed not only with cells such as SCC15 and HN886, which are moderately responsive to radiation, but also with SCC25, MSKQLL2, SCCVII cell lines, which are relatively resistant to radiotherapy.

The fraction of surviving cells was exponentially related to the dose of both the radiation and G207 (data not shown). Therefore, the product of the surviving fraction of cells after treatment with radiation and G207 represents the additive effect [15]. The dotted line in Fig. 1 reflects the hypothetical additive effect of an exclusive radiation therapy and an exclusive G207 virus therapy. In all six-cell lines, combination treatment significantly increased the cytotoxicity compared with either single therapy ($P < 0.01$). Furthermore, among five of the six cell lines (SCC1483, SCC25, MSKQLL2, HN886, SCCVII), the combination treatment effect was significantly better than the expected additive effect of the two therapies in combination (Fig. 1).

3.2. In vitro viral proliferation assay

A viral proliferation assay was performed to identify viral proliferation in the irradiated and non-irradiated cells after G207 viral therapy. The viral bursts in the SCC1483, SCC15 and SCC25 cell lines occurred between day 3 and 4 post-infection. The viral bursts in the MSKQLL2, HN886 and SCCVII cell lines occurred at day 1 post-infection. The viral bursts were not affected by radiation therapy. Increases in viral proliferation after radiation were noted in four of six cell lines. It was statistically significant in the SCC1483, SCC15, MSKQLL2 and SCCVII cell lines (repeated measured ANOVA, $P < 0.05$). Although viral proliferation did not increase significantly in the SCC25 and HN886 cell lines, it was not decreased in the irradiated group in comparison to the non-irradiated group (Fig. 2).

3.3. In vitro assay of the infection efficacy

The *In vitro* infection efficacy for the non-radiated and radiated cells was measured by histochemical staining for β-galactosidase (*lac*Z) with an X-gal solution 24 h after treatment with G207 at a MOI of 0.1. In some irradiated cell lines (SCC1483, SCC15, SCCVII), G207 demonstrated a significantly increased infection efficacy compared with non-irradiated cell lines, as determined by calculating the percent of *lac*Z-positive cells ($P < 0.05$), (Fig. 3). In the other cell lines (SCC25, MSKQLL2, HN886A), we detected a similar or slightly increased number of stained cells in the irradiated group compared with the non-irradiated ones. We found that low-dose irradiation (400 cGy) did not decrease the viral infectivity.

3.4. In vivo inhibition of tumour growth

S.c. SCCVII tumours were established in C3H/HeJ mice and treated with G207, with radiation therapy, and with both in combination. At day 21, intratumoral injections of G207 (1×10^7 pfu, two doses delivered 2 days apart) suppressed tumour growth up to 64% compared with controls, and low-dose radiation (500 cGy) suppressed tumour growth 30% compared with controls. Although either modality alone failed to halt tumour progression, the combination of G207 with radiation therapy demonstrated complete suppression of tumour growth. Combination therapy resulted in significantly reduced tumour volumes compared with controls or either single treatment modality. (Day 21 tumour volume: group I (control): 3540 ± 865 mm^3; group II (G207): 2488 ± 1431 mm^3; group III (RTx + PBS): 1205 ± 621 mm^3; group IV (G207 + RTx): 182 ± 165 mm^3; ANOVA and Tukey multiple comparison test, $P < 0.001$) (Fig. 4).

Similar experiments were performed in athymic nude mice using the SCC15 cell line. Tumour volumes for animals receiving the combination therapy of G207 and radiation were significantly reduced compared with controls or single treatment modalities. (Day 22 tumour volume: group I (control): 223 ± 114 mm^3; group II (G207): 124 ± 104 mm^3; group III (RTx + PBS): 47 ± 17 mm^3; group IV (G207 + RTx): 2 ± 2 mm^3; AN-

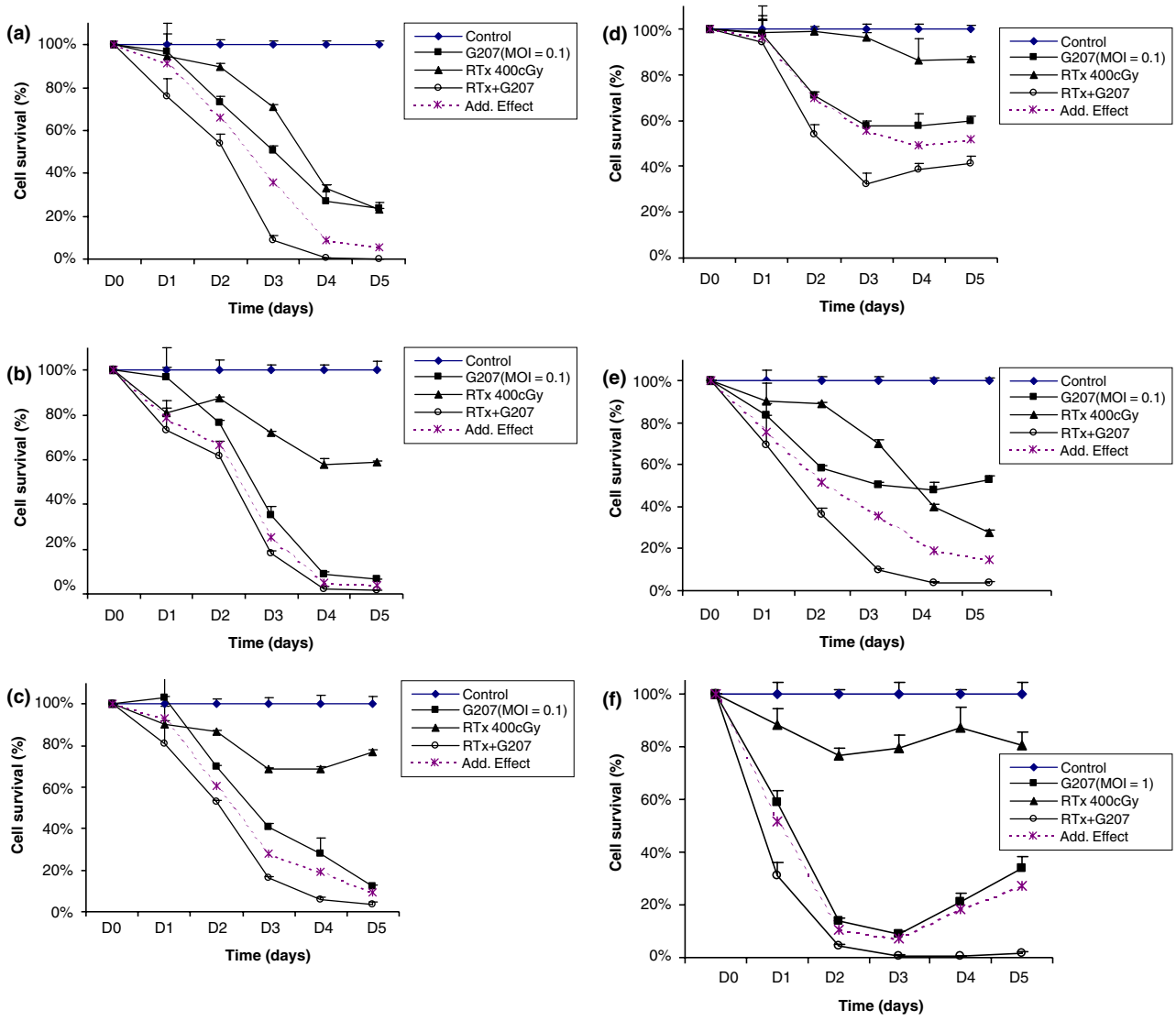

Fig. 1. *In vitro* cytotoxicity assays for the combination effect of G207 and radiation therapy on head and neck squamous carcinoma cell lines. The results of combining treatment are shown for SCC1483 (a), SCC15 (b), SCC25 (c), MSK QLL2 (d), HN886 (e), and SCCVII (f). The dotted line reflects the hypothetical additive effect of an exclusive radiation therapy and an exclusive G207 virus therapy. All tests were performed in quadruplicate. The product of the surviving fraction of cells after treatment with radiation and G207 represents the additive effect. MOI, multiplicity of infection; RT, radiotherapy.

OVA and Tukey multiple comparison test, $P < 0.05$) (Fig. 5).

3.5. In vivo viral proliferation assay

A viral proliferation test was done to identify viral proliferation in the irradiated C3H/HeJ syngeneic mice tumour tissue after a combination of radiation and G207 viral therapy. Viral replication was increased 4.3-fold on day 1 and increased 1.8-fold on day 2 in the irradiated tumour tissues in comparison to the non-irradiated tumour tissues (Repeated measured ANOVA, $P < 0.05$) (Fig. 6).

4. Discussion

The prognosis of patients with advanced stage head and neck squamous cell cancer remains poor despite recent advances using combined treatment modalities. This cohort of patients with advanced disease awaits the development of novel therapies to improve locoregional control, to increase survival, and to limit functional morbidity. Over the past 5 years, preclinical studies evaluating viral-vector-mediated treatments of head and neck squamous cell cancer have reported promising results [3–5,16]. One of these viral therapy strategies is oncolytic viral therapy. Carew et al. [16]

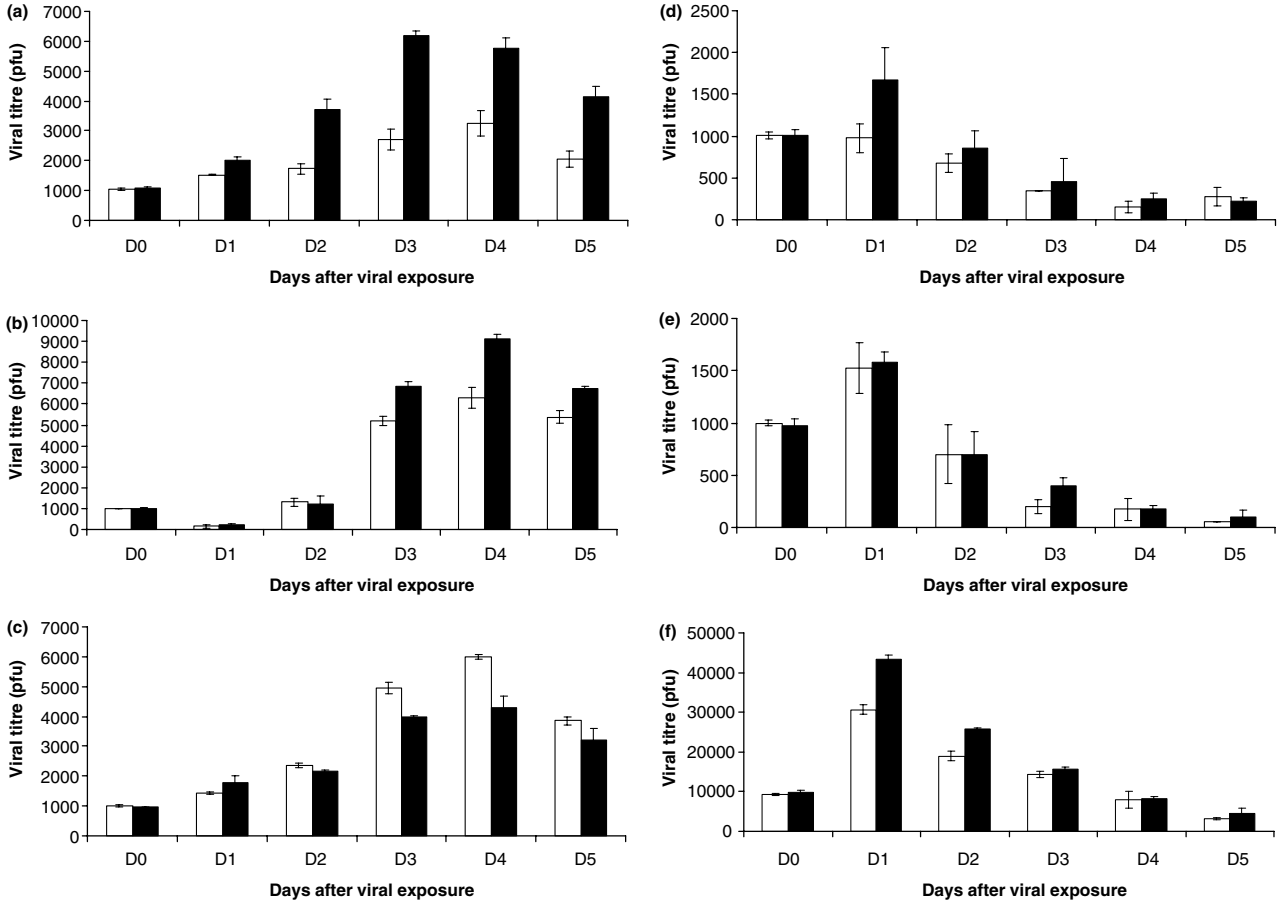

Fig. 2. *In vitro* viral proliferation assay for non-irradiated (open bars) and radiated (black bars) head and neck squamous carcinoma cell lines. The results of viral titres are shown for SCC1483 (a), SCC15 (b), SCC25 (c), MSK QLL2 (d), HN886 (e), and SCCVII (f). The viral burst in the six SCC cells lines occurred at similar periods and was not related to the radiation given. (Open bars (□) represent non-irradiated groups; black bars (■) represent irradiated groups). Pfu, plague-forming units.

and Wong et al. [17,18] have reported that the replication-competent herpes oncolytic virus, G207, has potent antitumour activity in a head and neck cancer model.

HSV have a natural affinity for neural tissue, and these vectors were originally designed for gene transfer into the nervous system [13,19]. The natural life cycle of the HSV involves an initial infection of a cutaneous or mucosal surface followed by a lytic replication cycle. Because of this natural ability to infect epithelial cells, it is not surprising that HSV vectors are efficient vehicles for gene transfer into epithelial-derived squamous carcinoma cells.

Oncolytic viral therapy uses genetically engineered viruses to kill tumour cells directly. An ideal virus for clinical use would have the following qualities: (1) avirulence to normal tissues; (2) replication competence; (3) potent oncolytic activity; and (4) susceptibility to existing antiviral therapies [20]. HSVs offer many advantages as oncolytic agents. The G207 virus is a multi-gene mutant of HSV-1, with deletions at both $\gamma_1$34.5 loci and a *lacZ* gene insertion in the ICP6 gene encoding the HSV ribonucleotide reductase [8]. The G207 virus is also highly sensitive to anti-herpetic drugs, and multiple mutations of the virus minimise the chance of spontaneous reversion back to the wild-type HSV-1. G207 causes no detectable disease in the brain [21,22]. The $\gamma_1$34.5 gene is responsible for much of the neurovirulence of HSV and is essential for the replication of HSV in the central nervous system because it prevents a cellular stress response that ordinarily occurs with the onset of viral DNA synthesis. Normally, $\gamma_1$34.5 prevent an inhibition of host protein synthesis, thereby permitting viral replication to continue. An absence of $\gamma_1$34.5 therefore results in an inhibition of viral replication. The $\gamma_1$34.5 genes of the HSV-1 genome block the phosphorylation of the α subunit of the translation initiation factor eIF-2, thus preventing a premature shutoff of protein synthesis [8]. The host protein shutoff has been mapped to the carboxyl terminus of the $\gamma_1$34.5 gene, and the amino acid at the carboxyterminus of the gene is homolo-

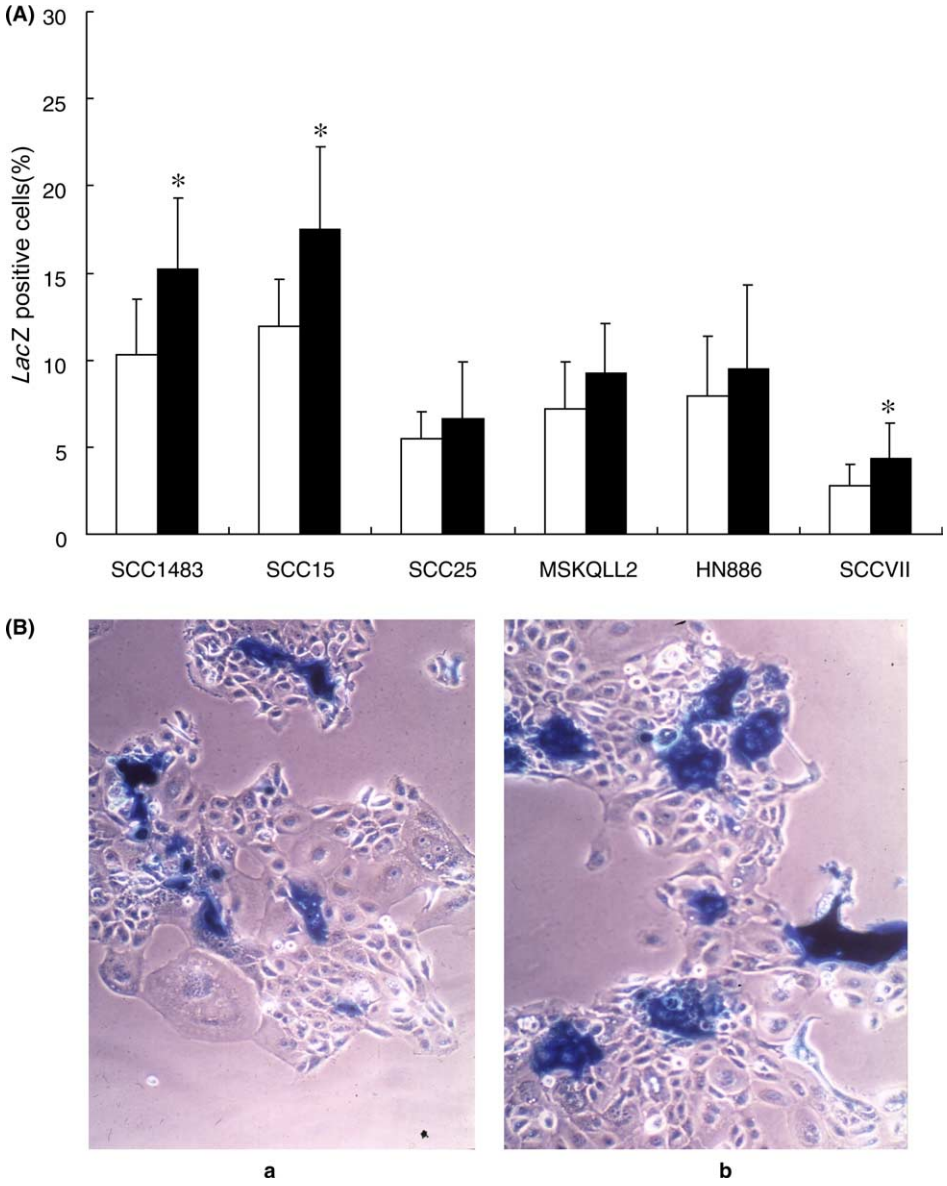

Fig. 3. Comparison of the *in vitro* infection efficacy between non-irradiated and irradiated cells by *lac*Z Staining. (A) The *in vitro* infection efficacy for non-radiated and radiated cells was measured by histochemical staining for β-galactosidase (*lac*Z) with an X-gal solution 24 h after treatment with G207 at a MOI of 0.1. All data represent the means ± standard deviations (SDs) of experiments performed in triplicate. (Open bars (□) represent the non-irradiated groups; black bars (■) represent the irradiated groups). $*P < 0.05$. (B) Lac-Z staining results in SCC15 cells. Control(a) and irradiated (400 cGy) cells (b); MOI = 0.1 (X100), $*P < 0.05$.

gous to a corresponding stretch of the murine protein, GADD34 [6].

Advani et al. [23,24] and Stanziale et al. [25] have reported an enhancement of replication of γ34.5-deleted herpes virus with radiation exposure. Blank et al. [26] and Jorgensen et al. [27] have also reported that the efficacy of G207 therapies was enhanced by radiation treatment in cervical and prostate cancers.

We hypothesised that the combination of radiation and G207 therapy would enhance the oncolytic activity of this γ34.5-deficient virus because of an upregulation of GADD34, while maintaining decreased neurovirulence. Our results confirmed that GADD34 expression was increased in head and neck cancer cell lines after radiation exposure (data not shown). In non-small cell lung cancer experiments conducted in our laboratory using short interfering RNA (siRNA) for GADD34, we confirmed that GADD34 was directly related with enhanced viral replication and cell kill (data not shown).

Another genetic alteration of G207 is the interruption of the ICP6 gene, which encodes the large sub-unit of the HSV ribonucleotide reductase. This loss of ribonucleotide reductase decreases its ability to proliferate in cells not undergoing rapid cell division. Ribonucleotide

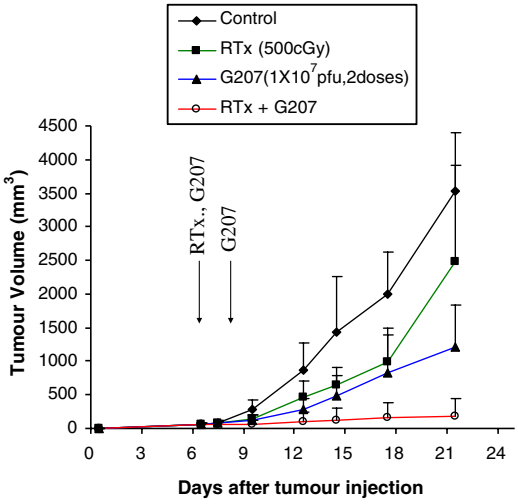

Fig. 4. *In vivo* tumour suppression assays for the combination effect of G207 and radiation therapy on the SCCVII syngeneic mice tumour model. RTx, radiotherapy.

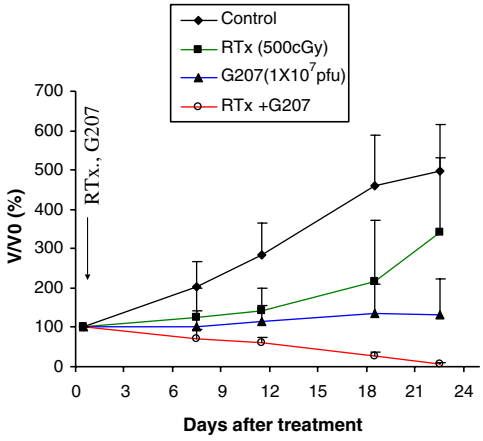

Fig. 5. *In vivo* tumour suppression assays for the combination effect of G207 and radiation therapy on the SCC15 xenograft tumour model.

reductase is the rate-limiting enzyme in *the de novo* synthesis of all deoxynucleotide triphosphates (dNTPs). It is known that a ribonucleotide reductase-deficient herpes virus may compensate for this deficit by utilising cellular ribonucleotide reductase [25]. Thus, rapidly dividing cells, which presumably express higher levels of ribonucleotide reductase, may serve as more suitable hosts for efficient G207 replication. Ribonucleotide reductase is highly regulated during S phase and after DNA damage to prevent dNTP misincorporation and to decrease the mutation frequency. Kuo and Kinsella [10] and Stanziale et al. [25] have reported an upregulation of ribonucleotide reductase and an increase in its activity after radiation of human cervical squamous cancer and a colorectal cancer cell model.

In the current study, both *in vitro* and *in vivo* viral growth studies demonstrated that a similar or increased viral replication occurs following radiation exposure of head and neck cancer cell lines. We speculated that both ribonucleotide reductase and GADD34 induction following DNA damage by radiation complemented with G207 were both responsible for the enhanced viral replication. In addition, we considered that mechanisms of G207 oncolytic therapy and radiation therapy are different. Generally, G207 infects and lyses cells, while radiation therapy induces DNA damage through apoptosis [28].

In vitro cytotoxicity assays demonstrated that the combination of G207 and radiation therapy displayed

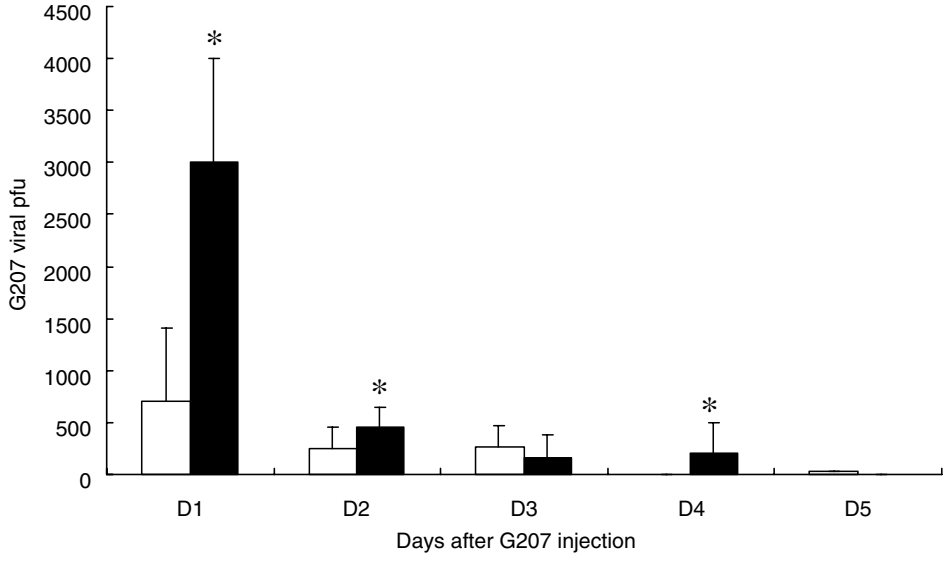

Fig. 6. *In vivo* viral proliferation assay for the combination effect of G207 and radiation therapy on the SCCVII syngeneic mice tumour model. SCCVII tumours *in vivo* were treated with either G207 (1×10^6 pfu) alone or with G207 with radiation (500 cGy). (Open bars (□) represent the non-irradiated groups; black bars (■) represent the irradiated groups; $^*P < 0.05$).

a more than additive effect in all six head and neck squamous cell carcinoma cell lines. During the entire 5-day period, the observed effect of the combination therapy was greater than the calculated additive effect. The effectiveness of this combination treatment was confirmed not only with cells such as SCC1483, SCC15 and HN886, which are responsive to radiation, but also with SCC25, MSKQLL2 and SCCVII cell lines, which are relatively resistant to radiotherapy.

In animal studies, the combination of G207 with radiation therapy resulted in nearly complete inhibition of tumour growth, while tumours treated with any single modality continued to progress ($P < 0.001$). The combination effect on cytotoxicity was more prominent in the *in vivo* experiments. Given the decrease in cell numbers due to cell death following radiation in the *in vitro* experiments, it is likely that viral replication was more efficient with radiation in live tumour tissues.

Advani et al. [23] reported that the combination of radiation with R3616 (a $\gamma_1$34.5-deleted HSV) was synergistic in the suppression of a malignant glioma xenograft tumour model. However, previous studies [23–25] investigating the combination of radiation and G207 viral therapy are problematic because the radiation doses used were much higher than those that are clinically relevant.

In this study, the amount of radiation used was similar to a clinically applicable dose and was shown to have an encouraging effect in combination with an oncolytic HSV for head and neck cancer. If we try to apply this combination strategy to the clinical situation, low doses of the radiation dose would decrease complications and are clearly desirable. We can expect that the effect may be transient in that once ribonucleotide reductase and/or GADD proteins cease to function, the cells become invulnerable to viral infection. For consecutive induction of these proteins, low-dose radiation may therefore be more suitable and desirable for clinical use. Further studies of the duration of expression and optimal concentration of these molecules for viral growth are needed.

In summary, the combination of low-dose radiation with oncolytic viral therapy has demonstrated a more than additive antitumoral effect, both *in vitro* and *in vivo* in treating HNSCC. In addition, viral replication was not adversely affected or increased by low-dose radiation. The superior efficacy of the combined treatment illustrated that the two treatments can individually exert their cytotoxic effects and can be successfully combined to improve the response to therapy. Radiation confers favourable conditions for G207 viral replication, perhaps explaining the more than additive effects for the combination therapy demonstrated in our study. Our results suggest that a combination of radiation and oncolytic HSV therapy may have the potential to enhance the treatment of radiation-resistant head and neck cancers.

Conflict of interest statement

None declared.

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