IMPACT OF NOVEL ONCOLYTIC VIRUS HF10 ON CELLULAR COMPONENTS OF THE TUMOR MICROENVIROMENT IN PATIENTS WITH RECURRENT BREAST CANCER

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Abstract

Oncolytic viruses are a promising method of cancer therapy, even for advanced malignancies. HF10, a spontaneously mutated herpes simplex type 1, is a potent oncolytic agent. The interaction of oncolytic herpes viruses with the tumor microenvironment has not been well characterized. We injected HF10 into tumors of patients with recurrent breast carcinoma, and sought to determine its effects on the tumor microenvironment. Six patients with recurrent breast cancer were recruited to the study. Tumors were divided into two groups: saline-injected (control), and HF10-injected (treatment). We investigated several parameters including neovascularization (CD31) and tumor lymphocyte infiltration (CD8, CD4), determined by immunohistochemistry, and apoptosis, determined by TUNEL assay. Median apoptotic cell count was lower in the treatment group (p=0.016). Angiogenesis was significantly higher in treatment group (p=0.032). Count of CD8-positive lymphocytes infiltrating the tumors was higher in the treatment group (p=0.008). We were unable to determine CD4-positive lymphocyte infiltration. An effective oncolytic viral agent must replicate efficiently in tumor cells, leading to higher viral counts, in order to aid viral penetration. HF10 seems to meet this criterion; furthermore, it induces potent antitumor immunity. The increase in angiogenesis may be due to either viral replication or the inflammatory response.

Key Words: oncolytic virus, HF10, herpes virus, breast cancer, angiogenesis, apoptosis, microenvironment

INTRODUCTION

Carcinoma of the breast is the most common cancer among females. Currently, 40% of breast cancer patients are predicted to suffer from either locoregional (isolated) recurrence or systemic metastasis. Ten to 20% of all recurrences are locoregional, whereas 60 to 70% are distant metastases.[1-3] Despite multimodal treatment, including chemotherapy and hormonal therapy, the prognosis for patients with recurrent or metastatic breast cancer remains poor.[4] Therefore, more specific, safe and effective treatment modalities are required. A growing body of preclinical and clinical data suggests that oncolytic viral therapy could be an effective therapeutic modality in the treatment of advanced cancer.[5-11]

Various strains of viruses, such as adenovirus[12], herpes simplex virus[13], Newcastle disease virus, measles virus, vesicular stomatitis virus, and vaccinia virus[14] are being analyzed for their oncolytic capacity; some of these viruses have progressed to the clinical trial phase. Herpes simplex virus type 1 (HSV 1) is an ideal candidate for oncolytic viral therapy because a) it infects a broad range of hosts; b) it causes lyses of the host cell at the end of viral replication; c) it has a very large genome and therefore harbors many non-essential gene, mostly related to neuroinvasiveness that are expendable and can be replaced during the recombinant engineering process; d) it can be controlled by antiviral drugs in the event of uncontrolled replication; and e) its genome remains as an episome and does not incorporate in to the host genome, avoiding the risk of introducing mutations.[15]

A unique and spontaneously mutated and naturally mutated HSV 1, HF10, has been demonstrated to be an effective oncolytic agent in preclinical contexts including peritoneal dissemination models, breast cancer xenografts, and malignant melanoma

models.[16-19] In all these studies, HF10 has been effective in tumor lyses. Previously, we published a promising preliminary clinical study demonstrating the efficacy of HF10 in patients with recurrent breast cancers or unresectable pancreatic carcinoma.[20,21] The power of HF10 lies in the fact that it is a spontaneous mutant and is genetically very similar to the parental virus.

The tumor microenvironment plays an important role in the survival, proliferation, and invasion of tumor cells.[22] The microenvironment is composed of non-transformed cells such as stromal, endothelial and immune cells, all of which are surrounded by the extracellular matrix (ECM).[23] In order to increase in size, tumor tissue requires formation of new blood vessels nearby.[24] Therefore, therapeutic modalities with an effect on angiogenesis are essential for successful cancer therapy. Preclinical data regarding herpetic stromal keratitis has revealed enhanced angiogenesis upon infection with to wild-type HSV.[25] The situation is more complicated for oncolytic viruses: some well-designed studies indicate that such viruses may have anti-angiogenic properties,[24,26] whereas other researchers have reported enhanced angiogenesis occurring via a range of mechanisms.[27-29] There are many attempts to emgineer viruses to express anti-angiogenic molecules.[30-32] Also obscuring the true effect of oncolytic herpes viruses on angiogenesis is the fact that all data have been generated in animal models rather than clinical investigation.

Oncolytic viral therapy of cancer has an advantage when compared to conventional cancer therapeutics, namely, the potential to induce antitumor immunity due to viral replication and oncolysis. A number of preclinical studies have shown that oncolytic herpes viruses induce cytotoxic T lymphocyte–mediated antitumor immunity, which can inhibit tumor regrowth upon rechallenge.[33-36] Consistent with this, efforts

have been made to arm oncolytic herpes viruses with cytokines such as IL-12[37] and GM-CSF[38, 39] in order to intensify antitumor immunity. From this perspective, it might be said that the potency of a virus relies on its capacity to induce antitumor immunity.

In cells, induction of apoptosis can be a protective mechanism against viral infections.[40] HSV 1 can reduce apoptosis via the ICP 34.5 gene product, which modifies the protein kinase R pathway, blocking the apoptotic mechanism in normal cells.[40-43] The effects of oncolytic herpes viruses on apoptosis are controversial: apoptosis is elevated upon viral treatment in some studies,[44,45] but reduced in others.[46,47] Some have argued that induction of premature apoptosis is not a desirable feature of oncolytic viral treatment[48]; instead, reducing or delaying apoptosis may enhance viral penetration of the tumor, another determinant of the potency of an oncolytic virus.

The value of the data presented in this study relies on the fact that it was generated in actual clinical samples rather than experimental animal models. Here we aimed to evaluate the cellular effects of HSV 1, a spontaneous oncolytic mutant of HSV 1, on the tumor microenvironment in patients with recurrent carcinoma tumors.

PATIENTS AND METHODS

HF10 virus

HF10 is a spontaneous mutant strain of HSV 1 whose mutations confine viral replication to cancer cells. The genome and genetic alterations of this herpes simplex virus are summarized in Figure 1.[20] Briefly, the virus carries two main genetic alterations; i) a 3832-bp deletion leading to loss of the UL56 promoter, making the gene dysfunctional; and ii) near the terminal redundance sequence in long arm (TRL) end of the genome, a 6027-bp segment is present in an inverted orientation. The loss of UL56 function is the major functional alteration.[16,49] This mutation significantly decreases the neuroinvasiveness of the virus, possibly by decreasing axonal vesicular transport. Nevertheless; the mechanism that confines the virus to cancer cells is not clearly understood.

Patients Characteristics

All the patients were female; their ages ranged from 48 to 76 years. All subjects were antibody-positive against HSV 1. Mastectomy had been performed on all of the patients, and all had received some treatment modality such as chemotherapy, endocrine therapy, surgical therapy, and/or radiotherapy. Despite such treatment, however, these patients had recurrences and the disease had progressed to metastasis to the superficial or the subcutaneous region of the skin (Figure 2). The clinical parameters of the patients are summarized in Table 1. No specimen from patient number 6 was available, due to extensive fibrosis and a lack of tumor cells in the pathology sample. This study was approved by the local ethics committee and by the institutional review board (IRB) of our hospital; all patients gave written informed consent.

Dosing Interval of the Virus

At least two tumor nodules were chosen; HF10 was injected into one nodule, and saline into the other as the mock control. The dosing regimen and the response to treatment for each patient are described in Table 2. In all patients, the first nodule (~1 cm in diameter) was injected with virus suspended in diluents at various doses, as follows: single dose injection of 10^4 pfu/0.5 ml to patient 1; single dose injection of 10^5 pfu/0.5 mL to patient 2; three-dose injection of 10^5 pfu/0.5 mL to patient 3; single dose injection of 5×10^5pfu/0.5 mL to patients 4 and 5; three-dose injection of 5×10^5 pfu/0.5 mL to patient 6 (Table 2). Three or four different sites of the tumor were injected in order to infiltrate HF10 into the entire nodule. The second nodule, located more than 5 cm from the first nodule, was injected with 0.5 mL of sterile saline following the same dosing intervals as the viral injection counterpart. The tumors were resected 14 days following the initiation of treatment. Histopathological responses were evaluated according to criteria established by the Committee for Production of Histopathological Criteria of the Japanese Breast Cancer Society.[50]

Histological Analysis

Standard hematoxylin–eosin staining was performed on 5-μm tissue sections for thorough histological analysis that included orientation of cells, herpetic inclusion bodies, and general cellularity of the specimen under 100× magnification.

Immunohistochemistry

5-μm thick serial sections were taken. Antigen retrieval was carried out with Tris/EDTA, pH 9.0, in an autoclave for 15 min. After blocking with 3% normal goat

serum (Histofine; Nichirei Biosciences Inc., Tokyo), sections were incubated overnight with primary antibodies against CD8 and CD31 (dilution for both was 1:100) (Abcam, Cambridge, MA). Biotinylated anti-rabbit IgG (Abcam, Cambridge, MA) was used as the secondary antibody. HRP -DAB (Abcam, Cambridge, MA) was used as the chromogenic agent, and hematoxylin as the counter-stain. Each slide was examined with a light microscope at 200–400× magnification. 6 random areas were chosen within each section, and the number of positive areas was counted for each area on each slide.

Colorimetric TUNEL Assay

TUNEL staining of tissue was carried out using a DeadEnd colorimetric apoptosis detection system, (Promega Corporation, WI). Briefly, slides were immersed in 100 µl Equilibration Buffer at room temperature for 5–10 minutes. TdT reaction mix (Promega Corporation, WI) was added onto the slides and incubated for 60 minutes at 37°C in a humidified chamber. The reaction was stopped by immersing the slides in 2× SSC (Promega Corporation, WI) for 15 minutes. Streptavidin HRP-DAB (Promega Corporation, WI), diluted 1:500 in PBS, was used as the chromogenic agent. Following the application of the cover slip, each slide was examined with a light microscope at 100× magnification. In both control and HF10 treated groups; apoptotic cell counts were determined in 6 random areas in similar large tumor regions with moderate to high cell counts. Therefore the results were expressed as counts per high power field.

Anti-HSV 1 Immunofluorescence

Polyclonal RabbitAnti-Herpes Simplex Virus Type 1/FITC conjugated antibody (DakoCytomation, Glostrup) was used to perform the immunofluorescence staining

according to the manufacturers' instructions. Briefly, slides were deparaffinized, air dried, and incubated with Polyclonal Rabbit Anti-Herpes Simplex Virus Type 1/FITC antibody (1:40 dilution) for 1 hour. Subsequently, slides were air dried, mounted and observed with a fluorescence microscope. The slides were evaluated at 100× magnification.

Statistical Analysis

Data are expressed as means and standard deviations. The Mann–Whitney U test was used to compare the data obtained from the study groups. Statistical analysis was performed using SPSS Statistics software, version 15.0 (SPSS Inc, IL, USA). Statistically significant difference was inferred when $P<0.05$.

RESULTS

Throughout the study; no adverse effects due to administration of HF10 were observed; all patients tolerated the therapy without any problem.[20]

During the follow-up period, we observed a 30–70% reduction in the size of the tumors treated with HF10.

Microscopically, the tumors treated with HF10 showed lower cellularity than mock-treated control tumors. The cells gradually shrank and fibrosis took over. In fact, in the patient 6, the fibrosis was so massive that no tumor tissue could be identified; this sample was therefore excluded from the histopathological analysis. HSV 1 inclusion bodies could be observed in the HF10-treated group; anti-HSV 1 immunofluorescence staining also confirmed the presence of the virus (Figure 4a). HSV antigen was detected at the tumor islands and not in normal tissue. Furthermore; the viral antigen was distributed throughout the tumor. The mock-treated control tumors did not express the antigen.

Mean apoptotic cell count was 25.6 per high power field (hpf) at 100× magnification in HF10-treated tumors, vs. 47.4/hpf in the control tumors (Figure 4b, P=0.016). The apoptotic bodies were scattered uniformly throughout the tumor islets.

As shown in Figure 4c, neovascularization (identified by CD31 staining) was significantly higher in HF10-treated tumors than in control tumors (mean of 30.0/hpf at 200× magnification, vs. 12.0/hpf; P=0.032). The neovascularization areas were more prominent at the junction between tumor and stroma. At higher magnification, we observed that some regions contained extensions into the tumor islets (Figure 4d).

Mean counts of CD8-positive lymphocytes infiltrating the tumors in the HF10-treated and control tumors were 75.0 and 42.0/hpf at 400× magnification, respectively (Figure 4e, P=0.008). CD8-positive T lymphocytes surrounded each tumor islet; as they infiltrated more deeply, the tumor cells became more hyperchromatic and shrunken in size, suggestive of a cytotoxic T cell–mediated antitumoral immune response. We were unable to detect CD4-positive cells in either HF10-treated or mock-treated tumors (Figure 4f).

DISCUSSION

Cancer is a complex and a multi-factorial disease. Following malignant transformation, cancer cells need an environment that is suitable for nourishment, proliferation, migration and invasion. The tumor microenvironment is very similar to sites of inflammation during the wound healing process, which promotes angiogenesis, turnover of the extracellular matrix (ECM), and tumor cell motility.[51] The tumor microenvironment consists of non-malignant cellular components such as fibroblasts, endothelial cells and immune cells. Therefore, in order to elucidate the mechanism of tumor lysis by oncolytic viral agents, it is important to understand the interaction between the viruses and the tumor microenvironment.

HF10 is a spontaneously occurring mutant virus isolated from the herpes simplex type 1 strain HF by Nishiyama et al.[52] The antitumor effects of HF10 are more potent than those of genetically engineered viruses, because HF10 is a spontaneously mutated virus. Our preclinical and preliminary clinical data suggest that HF10 is an effective agent for treatment of non-neurogenic tumors.[53] Before now, however, the effects of HF10 and other oncolytic herpes viruses on cellular components of the tumor microenvironment have not been well characterized. This is the first study to specifically analyze the effects of this virus on the tumor microenvironment following intratumoral injection in patients with recurrent breast cancer.

In this study, we observed that HF10-treated tumors had significantly lower apoptotic cell counts than mock-treated tumors. We believe that HSV 1 has a tendency to reduce apoptosis through several mechanisms, such as the PKR pathway and the US3 arm of the viral genome (54). Some oncolytic herpes viruses reduce apoptosis[46,47,55] ;

whereas others enhance apoptosis.[44,45,48] The effect of oncolytic herpes viruses on apoptosis is strain-specific and depends on the underlying genetic variation. Defects in the US3 arm or gamma 34.5 may induce apoptosis in infected cells; this may explain why gamma 34.5–deleted viruses such as G207 induce apoptosis. In such cases, the virus usually has a limited infection area and cannot spread throughout the tumor. In contrast to G207, HF10 spread throughout the whole tumor area, as demonstrated by staining for the HSV1 antigen. The fact that HF10 reduces apoptosis may be related to its ability to spread throughout the tumor instead of being confined only to the injection site. Eisenberg et al. reported that virus-related apoptosis in pancreatic cancer cell lines, which is induced by Hsp72, was reduced in hyperthermia; if virus-related apoptosis is reduced, *in vitro* viral titers and cytotoxicity will be increased.[56] These observations suggest that if viral infection causes apoptosis, the amount of infectious particles will gradually decrease. In a study of E1B55 attenuated adenovirus, Ganly et al. emphasized that virus-induced apoptosis was distinct from virus-induced cytolysis: apoptosis causes a premature cessation of viral replication, whereas cytolysis results in release of infective progeny.[57,58] In the present study; it may be hypothesized that lower cell counts in the HF10 treated group have caused the low apoptosis counts. However; we selected similar tumor areas with moderate to high cell number; for both control and HF10 treated groups; and performed the cell counts in 6 random fields in the same area. Therefore; we believe that our results in fact show that apoptosis is reduced by HF10.

We also observed that oncolytic viral therapy with HF10 enhanced angiogenesis, possibly due to the inflammatory response induced by viral infection, and viral proteins expressed during viral replication[30] The mechanisms underlying the enhanced

inflammation are not precisely known, but preclinical data regarding herpetic stromal keratitis in wild-type HSV 1 infection revealed that angiogenesis may be induced by paracrine effects resulting from release of VP22 or the CpG motifs in the DNA of HSV 1, which are required for viral replication.[59-62] Most of these stimuli, especially the CpG motif, potently stimulate secretion of VEGF A.[60-63] In addition, antiangiogenic molecules such as thrombospondin 1 and 2 are reduced in wild-type HSV 1 infection.[61] Many studies have shown that VEGF A is upregulated in HSV 1 infections[64-66]; thus, VEGF A may be the factor underlying the angiogenesis seen in our study.

Angiogenesis induces tumorigenesis. One study by Florence et al. showed that angiogenesis is higher in invasive cutaneous squamous cell cancers than in carcinoma *in situ* or microinvasive carcinoma.[67] In addition, a number of studies have shown beneficial effects of antiangiogenic treatment in various cancer models.[68-71] The angiogenesis caused by oncolytic herpes viruses may have deleterious effects on the late phase of cancer therapy, especially with regard to late recurrences that occur after the end of the treatment regimen. In order to investigate this effect, we initiated a series of preclinical studies combining oncolytic herpes viruses with the monoclonal anti-VEGFA antibody bevacizumab in various cancer models.

Our results suggest that treatment with HF10 induces a cytotoxic T lymphocyte response directed against the tumor. This has been supported by many studies of oncolytic viruses including HF10.[17,19,33-35] Furthermore, HF10 induces antitumor immunity more efficiently than hrR3 which is also an HSV 1 variant.[17] Oncolytic replication of a virus is an immunogenic event[71] that generates a response against both viral and tumor antigens.[72,73] Herpes simplex viruses induce antitumor immunity by

activation of dendritic cells via Toll-like receptors 2 and 9, which in turn enhance antigen presentation and specific T and B lymphocyte responses.[74-76] In addition, herpes simplex virus reduces the number of myeloid-derived suppressor cells (MDSC), which contribute to tumor cells' ability to circumvent host immune surveillance.[63] This effect is possibly due to reducing the effects of induced the expression of VEGF A on vascular endothelial growth factor receptor 2 through soluble neuropilin-1.[63] Another explanation of the enhanced immune response may be the syncytial cytopathic effect induced by HF10, which is a very potent immune enhancer.[53,77,78] Today we are certain that every oncolytic virus induces a certain level of antitumor immune response, yet the potency of the response determines the efficacy of the virus.

In summary, the clinical data obtained in this study show that HF10 is a powerful oncolytic virus. By reducing apoptosis, it can thoroughly penetrate the tumor; furthermore, it induces a potent antitumor immune response that results in an efficient reduction of tumor volume. Its enhanced oncolytic activity is owed in part to the fact that it is a spontaneous, rather than engineered, mutant. All of the aforementioned factors, and the cytopathic effect of the virus contribute to the potent antitumor immunity caused by HF10. These characteristics make HF10 a potent, safe, promising oncolytic agent for the treatment of advanced carcinoma.

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CONFLICT OF INTEREST

We declare no conflict of interest for the present study.

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FIGURE LEGENDS

Figure 1 Summary of deletions and insertions in HF10's genomic structure. The expansions indicate the position of genes within the deletion, insertions, and relocalization. Arrows indicate the locations of the genes within expansions. **bp:** base pairs; **TRL:** Terminal Repeat Long; **TRS**: Terminal Repeat Short; **US:** Unique Short; **IRS:** Internal Repeat Short; **UL**: Unique long

Figure 2 Typical local recurrences, observed in a patient (arrows)

Figure 3 Hypocellularity is a common feature of HF10-treated tumors

Figure 4 (a)Herpetic inclusion bodies (arrow) are shown, as well as immunofluorescence staining. (b)The apoptotic cell count was reduced in the HF10-treated tumors (arrows). (c)CD31-positive microvascular density is enhanced in the HF10-treated tumors. (d)At closer magnification, it is clear that in some areas the neovascularization extended into the tumor islets (arrows). (e)CD8-positive lymphocyte infiltration is observed higher in tumors treated with HF10 than in the mock-treated tumors. (f)CD4-positive cells were not detected in any group. **TUNEL**: Terminal deoxynucleotidyl transferase dUTP Nick End Labeling

Table 1 The characteristics of the patients before the treatment.

Patient No.	Age	Histopathologic confirmation	Site of recurrence	Prior therapy	Interval from mastectomy (years)
1	61	Invasive ductal carcinoma	Skin, LN,brain, lung	CT,HT,RT	3
2	62	Invasive ductal carcinoma	Skin,LN	CT,RT	1.5
3	48	Invasive ductal carcinoma	Skin, LN,lung,bone	S	6
4	66	Invasive ductal carcinoma	Skin,LN	CT, HT	3
5	72	Mucinous carcinoma	Skin	S,CT,HT	24
6	76	Scirrhous carcinoma	Skin	CT,HT	7

LN: lymph node metastasis **MRM**:modified radical mastectomy **RM**: radical mastectomy

CT: chemotherapy **HT**:hormonotherapy**RT**: radiotherapy **S**: local excision

Table 2 The dosing regimens of HF10 and response of the patients to the treatment**.**

Patient No.	1. Dose	2. Dose	3. Dose	Response *
1	10^4 pfu/0.5 ml	-	-	Grade 1b
2	10^5 pfu/0.5 ml	-	-	Grade 1a
3	10^5 pfu/0.5 ml	10^5 pfu/0.5 ml	10^5 pfu/0.5 ml	Grade 2
4	$5x10^5$ pfu/0.5 ml	-	-	Grade 1b
5	$5x10^5$ pfu/0.5 ml	$5x10^5$ pfu/0.5 ml	$5x10^5$ pfu/0.5 ml	NA
6	$5x10^5$ pfu/0.5 ml	$5x10^5$ pfu/0.5 ml	$5x10^5$ pfu/0.5 ml	Grade 2-3

Pfu: plaque forming unit **ml**: milliliter

***** According to criteria of Committee for Production of Histopathological Criteria of the Japanese Breast Cancer Society[50]