Intratumoral Coadministration of Hyaluronidase Enzyme and Oncolytic Adenoviruses Enhances Virus Potency in Metastatic Tumor Models

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Abstract

Purpose: Evaluate the codelivery of hyaluronidase enzyme with oncolytic adenoviruses to determine whether it improves the spread of the virus throughout tumors, thereby leading to a greater overall antitumor efficacy in tumor models.

Experimental Design: The optimal dose of hyaluronidase that provided best transduction efficiency and spread of a green fluorescent protein (GFP)-expressing adenovirus within tumors was combined with oncolytic adenoviruses in tumor models to determine whether the combination treatment results in an improvement of antitumor efficacy.

Results: In mice injected with the adenovirus Ad5/35GFP and an optimal dose of hyaluronidase (50 U), a significant increase in the number of GFP-expressing cells was observed when compared with animals injected with virus only ($P < 0.0001$). When the oncolytic adenoviruses Ad50V or Ad5/35OV (OV-5 or OV5T35H) were codelivered with 50 U of hyaluronidase, a significant delay in tumor progression was observed, which translated into a significant increase in the mean survival time of tumor-bearing mice compared with either of the monotherapy-treated groups ($P < 0.0001$). Furthermore, the mice that received the combination of Ad5/35 OV and hyaluronidase showed the best antitumor efficacy. Importantly, the combination treatment did not increase the metastatic potential of the tumors. Lastly, the increase in virus potency observed in animals injected with both enzyme and virus correlated with enhanced virus spread throughout tumors.

Conclusion: Antitumor activity and overall survival of mice bearing highly aggressive tumors are significantly improved by codelivery of oncolytic adenoviruses and hyaluronidase when compared with either of the monotherapy-treated groups, and it may prove to be a potent and novel approach to treating patients with cancer.

The limited ability of Ad5-based oncolytic viruses to infect tumor cells efficiently and to spread through solid tumors may hamper their effectiveness in the clinic (1, 2). Due to variable expression levels of the coxsackievirus-adenovirus receptor on tumor cells, the surface receptor used by Ad5 viruses to bind and enter cells, the initial transduction of tumor cells by Ad5-based oncolytic adenoviruses is limited (3–7). By replacing the Ad5 fiber knob with the Ad35 knob, which recognizes the highly abundant CD46 receptor on tumor cells, tumor cell transduction is significantly improved (7, 8).

The efficient viral entry of Ad5/35 fiber-chimeric viruses correlates well with their improved antitumor efficacy in multiple different xenograft models compared with Ad5-based oncolytic adenoviruses (7). Although the fiber-chimeric viruses enter tumor cells more effectively than Ad5-based viruses, the inability of both of these viruses to spread efficiently throughout a tumor mass remains a major obstacle to the development of a successful adenovirus-based therapy for solid tumors (9). Based on recent studies, connective tissue and extracellular matrix (ECM) components apparently have a prominent role in inhibiting viral spread after intratumoral administration of adenovirus (1, 9–11), and previously published data suggest that there is enhanced viral distribution within the tumor when ECM is degraded by proteolytic enzymes such as trypsin or collagenase (12, 13).

Hyaluronan is a high molecular weight nonsulfated glycosaminoglycan polymer that is an important constituent of the ECM surrounding cancer cells where it forms large networks with proteoglycans (14). By degrading the hyaluronan-rich matrix with hyaluronidase, a naturally occurring enzyme that depolymerizes the viscoelastic ECM components, the barrier for drug/virus dispersion throughout the interstitial spaces may be improved (14, 15). The degradation of hyaluronan is likely to alter the configuration of the ECM and, thereby, to reduce interstitial fluid pressure, which is another obstacle to efficient spread of therapeutic agents during tumor treatment (16).

Recombinant human hyaluronidase enzyme (rHuPH20) has been developed and evaluated by Halozyme Therapeutics, Inc.
(17). Their studies have shown that rHuPH20 increases the transduction efficiency of an adenovirus expressing either green fluorescent protein (GFP) or the therapeutic gene PDGF-B in mouse skin (17).

To test the effects of hyaluronidase on virus transduction and spread in solid tumors, we selected two distinct but highly metastatic tumor models: PC-3.luc (human prostate) and A375-mln1.luc (human melanoma). Both of these models were used previously to test the effect of relaxin expressed by oncolytic viruses on virus spread, and we were interested to evaluate hyaluronidase coinjected with oncolytic viruses in these same models (11). Furthermore, PC-3 tumors have high levels of hyaluronan, and it was previously shown that expression of hyaluronan biosynthetic and processing enzymes promote growth and vascularization of PC-3 tumors in mice, thereby stimulating tumorigenicity and metastasis (18, 19). Tumor-produced hyaluronidase has also been shown to degrade hyaluronic acid into smaller oligomeric units and may possibly promote metastasis by inducing angiogenesis (18–24). Interestingly, hyaluronan and hyaluronidase have been detected in the invasive areas of highly aggressive PC-3 tumors (18–24). A375-mln-1, a human melanoma model, is another highly metastatic tumor model, and overexpression of hyaluronidase in human melanoma cell lines has been shown to induce vascularization of tumors in mice (20). On the other hand, literature suggests that eradication of a hyaluronan-rich environment by i.v. administration of hyaluronidase to severe combined immunodeficient mice bearing human tumor xenografts could reduce the aggressiveness of tumors, thereby potentially offering a new anticancer strategy (14, 25, 26). Based on the above findings and the important role of hyaluronidase as a matrix degradative protein, we hypothesized that rHuPH20 might facilitate spread of adenovirus within tumors due to the removal of the ECM that may act as a naturally occurring structural barrier to virus spread within tumors.

We have coadministered rHuPH20 with a fiber-chimeric E1-deleted adenovirus expressing GFP (Ad5/35GFP) to animals bearing human prostate PC-3.luc tumors, monitored GFP expression within the tumors, and compared it to tumors injected with either virus or rHuPH20 alone. These studies show that rHuPH20, when coadministered with adenovirus, enhances virus spread throughout the tumor when compared with virus treatments alone.

To determine if the improved spread of these adenovirus vectors in the presence of enzyme translates into improved antitumor efficacy and survival, the PC-3.luc and A375-mln1.luc xenograft models were used to test oncolytic adenoviruses and rHuPH20 for their effects on survival. The adenoviruses used in these studies are the oncolytic virus OV-5 (containing the original Ad5 fiber knob) and OV-5T35H (containing the Ad35 knob; ref. 7). In both viruses, the tumor-specific E2F-1 promoter replaces the native E1a promoter to restrict viral replication to cells that have a defect in the pRb pathway (27, 28). In addition, these viruses carry the cDNA encoding human granulocyte-macrophage colony-stimulating factor in place of the open reading frame encoding the viral 19-kDa glycoprotein in the E3 region. With the aim of improving viral entry and spread throughout the tumor, the Ad5 fiber knob of OV-5 has been replaced with the Ad35 knob to generate OV-5T35H (7).

**Materials and Methods**

**Nonreplicating and replicating viruses.** Ad5/35GFP is an E1-deleted, nonreplicating virus in which a GFP reporter gene, under the control of a strong constitutive cytomegalovirus promoter, has been incorporated into the E1 region of an adenovector that contains the fiber knob from Ad35 and the fiber shaft from Ad5 adenovirus (7). Oncolytic viruses OV-5 and OV-5T35H are conditionally replication-competent adenoviruses that were engineered to replicate selectively in tumor cells (7, 11). In these viruses, the tumor-specific E2F-1 promoter replaces the native E1a promoter to restrict viral replication to cells that have a defect in the pRb pathway (27, 28). In addition, the cDNA encoding human granulocyte-macrophage colony-stimulating factor replaces the open reading frame encoding the viral 19-kDa glycoprotein in the E3 region. With the aim of improving viral entry and spread throughout the tumor, the Ad5 fiber knob of OV-5 has been replaced with the Ad35 knob to generate OV-5T35H (7).

**In vivo efficacy studies.** Female NCR (nu/nu) mice (ages 4–6 wk; body weight, 18–20 g) were purchased from Simonsen Laboratories. Mice were injected s.c. in the right flank with either 5 × 10⁶ PC-3.luc cells or 2 × 10⁶ A375-mln1.luc cells (injection volume of 100 μl).

When the PC-3.luc tumors reached the desired mean tumor volume (125–150 mm³) as determined using the formula, Volume = W × L²/2, wherein W is width and L is length in millimeters, animals were randomly distributed into treatment groups (n = 3) and injected intratumorally with rHuPH20 enzyme (recombinant human hyaluronidase from Halozyme Therapeutics, Inc.) in PBS at a dose of 800 U per mouse per treatment. Ad5/35GFP virus at a dose of 1 × 10¹⁰ viral particles (vp in PBS), or a combination of Ad5/35GFP virus (1 × 10¹⁰ vp) and rHuPH20 enzyme at a dose of 50, 200, or 800 U in 50 μl PBS. Three days after virus injection, the tumors were harvested and analyzed in vivo for GFP-positive cells. Specifically, 5-μm serial sections were generated throughout the tumors and analyzed to select the section with peak GFP expression for each tumor. From the peak GFP expressing section, every third serial section in both directions (total of 12 sections per tumor) was selected, and a quadrant (consisting of 4 fields) in the center of these sections was analyzed at a ×10 magnification for the number of GFP-positive cells using an image analysis software program (Image Pro Plus; Media Cybernetics). The average number of GFP-expressing cells per field was determined from 3 animals per group, analyzing a total of 144 fields per group.

For efficacy studies, mice bearing established PC-3.luc (100–150 mm³ or ~250 mm³) or A375-mln1.luc (100 mm³) tumors received four or six intratumoral injections (50 μl) of PBS, OV-5 or OV-5T35H virus (1 × 10¹⁰ vp), rHuPH20 enzyme (50 μl per mouse), or a combination of virus and rHuPH20, once every other day. Tumor progression was monitored with survival as the end point. Animals were euthanized when tumor volumes reached 2,000 mm³ or if the antitumor activity and survival of tumor-bearing animals in both models are significantly improved by combination treatment with virus and enzyme, compared with treatment with either virus or enzyme alone. Moreover, these viruses in conjunction with enzyme do not increase the metastatic spread of tumor cells in a highly metastatic model. Such combination treatment may therefore offer a novel and improved oncolytic therapy for patients with cancer.
tumors became necrotic. Animals from each group were selected at various time points after the initial treatment, bled, euthanized, and tumors collected. Tumors were cut into halves, with one half used to determine viral spread by immunohistochemistry and the other half used to detect virus replication. For all in vivo efficacy studies, each group consisted of at least 10 mice. Tumor volume between different groups was compared by linear regression analysis. Survival times of mice from different groups was compared by Kaplan-Meier analysis.

For histologic examination, organs were collected in 4% paraformaldehyde in PBS and left in the solution for 3 h for complete fixing. The samples were then transferred to 30% sucrose solution for cryoprotection and left overnight at 4°C. Samples were rinsed in PBS, blotted dry, and blocks of respective tissues were embedded in omithine carbamyl transferase (STAT Lab Medical Products) before freezing in a methyl butane/dry ice bath. Five-microliter serial sections were collected and directly analyzed for GFP expression using fluorescence microscopy. GFP-positive cells in the tumors was quantified by determining the average number of GFP-positive cells per 10 field of view using an image analysis software program (Image Pro Plus; Media Cybernetics). To detect the hexon-positive cells, 5-μm paraffin sections of tumor tissue were stained for the adenoviral hexon protein using Zymed’s Histomouse kit following the manufacturer’s instructions (11). The remaining half of the tumor was used to isolate virus, and the titer was determined by infecting AE1-2a cells with the recovered virus (7, 11). To monitor metastasis of tumor cells, 6 lymph nodes (LN), including the axillary and inguinal nodes, and the lungs were collected at the selected time points, microinjected with luciferin substrate (15 mg/mL), and imaged after 2 min using the Xenogen imaging system (Xenogen Corp) to detect luciferase-positive metastases (11, 31).

Results

Intratumoral coadministration of hyaluronidase enzyme with Ad5/35GFP fiber-chimeric adenovirus enhances virus transduction/spread within established prostate cancer (PC-3.luc) tumors. To determine whether degradation of the ECM component hyaluronan by hyaluronidase enhances virus transduction of solid tumors, Ad5/35 E1-deleted adenovirus-expressing GFP was tested in combination with rHuPH20 in the PC-3.luc xenograft model. In this model, female nude mice were injected s.c. with 5 × 10⁶ PC-3.luc cells. Once the tumors reached a volume of 125 to 150 mm³, tumor-bearing mice (n = 3) were injected intratumorally with either Ad5/35GFP virus at a dose of 1 × 10¹⁰ vp or rHuPH20 enzyme at a dose of 800 U per mouse, or a combination of Ad5/35GFP virus (1 × 10¹⁰ vp) and rHuPH20 enzyme at doses ranging from 50 to 800 U. Three days postinjection, the tumors (n = 3) were harvested and the number of GFP-positive cells evaluated in tissue sections by immunohistochemical analysis as described in material and methods. An average of 79.2 GFP-positive cells per field was found in tumors injected with virus plus hyaluronidase compared with only 15.5 GFP-positive cells per field in tumors injected with virus alone (Fig. 1). The two higher doses of the enzyme (200 or 800 U) coincided with virus results in averages of 35.2 and 20.2 GFP-positive cells per field, respectively. These results confirmed that virus coinfected with 50 U of hyaluronidase leads to a more widespread distribution of virus within tumors than virus injected alone or virus injected with higher doses of the enzyme, suggesting that although spread of virus seemed to be enhanced with the two higher doses of hyaluronidase, it was optimal with the 50 U dose of the enzyme and, thus, this dose was considered the optimal dose of the enzyme to increase virus spread throughout the tumor and selected for further studies.

Intratumoral hyaluronidase coinjection with fiber-chimeric oncolytic adenovirus enhances the antitumor efficacy of the virus in the PC-3.luc model. To determine if the increased virus transduction observed with the combination of E1-deleted adenovirus and hyaluronidase in PC-3.luc tumors translated into enhanced efficacy, the fiber-chimeric oncolytic adenovirus was evaluated either as monotherapy or in combination with hyaluronidase in the PC-3.luc xenograft model with survival as the end point.

Nude mice bearing 100 to 150 mm³ PC-3.luc tumors received a total of four injections (50 μL intratumorally), given once every other day, of PBS, OV-5T35H (1 × 10¹⁰ vp per injection), rHuPH20 enzyme (50 U per injection), or the combination of these same doses of virus and enzyme. Tumor progression was monitored over time with survival as the end point. None of the animals exhibited any symptoms of toxicity or body weight loss as a result of any of the treatments (data not shown).

In the groups treated with either PBS or enzyme alone, tumor volumes increased by 9.3- and 11-fold, respectively, over a period of 33 days. In the group treated with OV-5T35H alone,

Fig. 1. Coadministration of recombinant hyaluronidase increases the spread of Ad5/35GFP virus in the PC-3.luc model. Nude mice (n = 3 per group) bearing s.c. PC-3.luc tumors of ~150 mm³ were injected intratumorally with either hyaluronidase (rHuPH20; 800 U per tumor) or Ad5/35 virus (1 × 10¹⁰ vp) or a combination of Ad5/35GFP virus (1 × 10¹⁰ vp) and rHuPH20 at doses of 50, 200, or 800 U. Three days postinjection, tumors were collected and GFP expression evaluated in tissue sections by immunohistochemical analysis. (magnification, ×5). The number of GFP-positive cells was quantified by counting all positive cells in a central quadrant (4 fields) of 12 tissue sections localized at the area of peak GFP expression and averaging them from 144 evaluated fields using an image analysis software program (Image Pro Plus; Media Cybernetics).
the tumor volume increased by only 3.3-fold (Fig. 2A), whereas in the group treated with the combination of virus and enzyme, the tumor volume increased only 1.3-fold during the same time frame. These data suggest that the fiber-chimeric oncolytic virus in combination with rHuPH20 was more efficacious than the virus alone. Complete tumor regression was noted in 2 of 10 mice treated with either OV-5T35H alone or with OV-5T35H and enzyme.

Animals treated with PBS alone had a mean survival time (MST) of 38.5 days with no animals alive by day 62, and animals treated with rHuPH20 alone had a MST of 35 days with no animals alive by day 69. In contrast, animals treated with OV-5T35H alone survived significantly longer than PBS or rHuPH20 injected animals with a MST of 65.5 days (OV-5T35H versus rHuPH20 or PBS; \( P < 0.01 \)). Animals treated with both OV-5T35H virus and rHuPH20 showed a further improvement in survival with a MST not yet reached by day 115 (OV-5T35H+rHuPH20 versus OV-5T35H; \( P < 0.05 \); Fig. 2B).

To determine if this potent combination therapy of virus and enzyme could also control larger PC-3.luc tumors, animals with established PC-3.luc tumors at a size of \( \approx 250 \) mm\(^3\) were treated with a total of 6 injections (50 \( \mu \)L intratumorally), given once every other day, of PBS, OV-5T35H virus (\( 1 \times 10^{10} \) vp per injection), rHuPH20 enzyme (50 U per injection), or a combination of OV-5T35H and rHuPH20, and animals were monitored over time to determine survival. Animals treated with the combination of OV-5T35H plus hyaluronidase showed a statistically significant survival advantage (\( P < 0.01 \); MST not reached) compared with animals treated with PBS (MST, 40 days), rHuPH20 (MST, 38.5 days), or virus alone (MST 50, days), thereby emphasizing the potency of this combination therapy, even in animals with a very large tumor burden (data not shown).

**Intratumoral hyaluronidase coinjection with oncolytic adenovirus enhances the antitumor efficacy of the virus in A375-mln1.luc tumor-bearing mice.** The combination of oncolytic virus and rHuPH20 enzyme was further tested in the highly metastatic A375-mln1.luc xenograft model to evaluate the potency of the virus and enzyme combination in another tumor model. Both the parental Ad5-based OV-5 virus and the fiber-chimeric OV-5T35H virus were tested in the A375-mln1.luc model because the Ad5-based virus transduces this tumor less efficiently than the chimeric virus (11), and thus, this model can test the effect of rHuPH20 treatment on tumor transduction by two oncolytic viruses with very different potencies.

Nude mice were injected s.c with \( 2 \times 10^6 \) A375-mln1.luc cells. When the tumors reached a volume of 100 mm\(^3\), tumor-bearing mice received a total of 4 intratumoral injections (50 \( \mu \)L intratumorally), given once every other day, of PBS, OV-5 or OV-5T35H (\( 1 \times 10^{10} \) vp per injection), rHuPH20 (50 U per injection), or a combination of either virus and enzyme. None of the animals exhibited any symptoms of toxicity or body weight loss as a result of these treatments (data not shown).

In the groups treated with PBS or enzyme alone, the tumor volume increased 6-fold over a period of 22 days (Fig. 3A). In contrast, in the group treated with either oncolytic virus alone or in combination with enzyme, there was a significant delay in tumor progression. Specifically, in the OV-5–treated mice, the tumor volume increased 5-fold, whereas in the OV-5T35H–treated animals, tumor volume increased only 3.5-fold during the same time period (OV-5T35H versus OV-5; \( P < 0.05 \)). When OV-5 virus was combined with rHuPH20, the overall rate of tumor progression decreased compared with OV-5 treatment alone, with a 3.5-fold increase in tumor volume, which is similar to the increase in tumor volume observed with OV-5T35H monotherapy. When mice were treated with OV-5T35H virus and enzyme, tumor progression was further delayed compared with the OV-5T35H–treated group (\( P < 0.0001 \)) as well as to the OV-5 plus enzyme-treated group (\( P < 0.0001 \)), exhibiting an increase in tumor volume of 1.2-fold by day 22 (Fig. 3A). Complete tumor regression was noted in 2, 4, and 5 of 15 mice treated with either OV-5T35H, OV-5 with enzyme, or OV-5T35H with enzyme, respectively, by day 65. As described above, tumor progression rates correlated with
improved survival. Animals treated with PBS or enzyme had MSTs of 24 and 27 days (Fig. 3B), whereas animals treated with OV-5 monotherapy had a MST of 40 days. In contrast, animals treated with either OV-5T35H monotherapy or the combination of OV-5 and enzyme survived longer, with MSTs of 45 and 47.5 days, respectively. Lastly, animals treated with OV-5T35H and enzyme had not reached the MST by day 65, and 67% of the animals remained alive at that time point.

In summary, the tumor growth rate and survival data confirmed that the combination of oncolytic adenovirus and rHuPH20 has greater antitumor efficacy compared with virus monotherapy in animals bearing highly metastatic A375-mln1.luc tumors. Furthermore, the enzyme combined with the fiber-chimeric oncolytic virus OV-5T35H provided the most efficacious treatment with a statistically significant survival advantage over animals treated with any of the other therapies.

**Improved viral spread and replication in the tumor underlie the enhanced potency of oncolytic adenovirus when coinfected with rHuPH20.** To understand the mechanism underlying the increased therapeutic efficacy and survival observed with the combination treatment of oncolytic adenovirus and rHuPH20, the number of adenoviral hexon-positive cells and infectious virus particles within the tumors was determined in the above study.

Tumors were harvested on day 10 after initiation of treatment, and the number of hexon-positive cells was roughly estimated by evaluating immunohistochemical stainings (Fig. 4A). Staining and evaluation of hexon-positive cells in tumor sections (magnification, ×10) showed that tumors treated with the combination of virus and enzyme had more hexon-positive cells compared with tumors treated with the parental or chimeric oncolytic virus alone. Hexon-positive cells also seemed to be lower in number in tumors treated with OV-5, either as monotherapy or in combination with enzyme, compared with tumors treated with OV-5T35H monotherapy or OV-5T35H in combination with rHuPH20. No hexon-positive cells were found in control tumors injected with either PBS or enzyme alone (data not shown).

To evaluate whether an increase in hexon-positive cells correlates with the presence of higher amounts of infectious virus within tumors, infectious virus particles in tumors were measured by titrating tumor homogenates on AE1-2a cells (7). No significant difference in the amount of infectious virus was found in tumors from animals treated with either OV-5T35H or the combination of OV-5 and rHuPH20. However, there was 10-fold less infectious virus present in tumors treated with OV-5 compared with tumors treated with either OV-5T35H alone or the combination of OV-5 and enzyme. Only a small increase in the level of infectious virus was detected in tumors treated with the combination of OV-5T35H and enzyme compared with tumors treated with OV-5T35H alone (Fig. 4B).

Thus, the degree of virus spread and number of infectious particles in the A375-mln1.luc tumors following the different treatments correlated well with antitumor efficacy in vivo, suggesting that extensive virus spread and a high level of infectious virus particles in tumors resulted in reduced tumor progression and increased overall survival of tumor-bearing animals.

**Fiber-chimeric oncolytic virus coinfected with hyaluronidase controls metastases in A375-mln1.luc tumor bearing mice.** One potential concern with hyaluronidase injections into tumors was whether cotreatment with the enzyme might increase the metastatic spread of the primary tumor. To address this concern, hyaluronidase only or in combination with the chimeric virus was injected into A375-mln1.luc tumors and metastatic spread of tumor cells to LN and lungs was monitored. Specifically, established A375-mln1.luc tumors of ~100 mm³ received a total of 4 injections (50 µL intratumorally), once every 47.5 days, respectively. Lastly, animals treated with OV-5T35H and enzyme had not reached the MST by day 65, and 67% of the animals remained alive at that time point.

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Fig. 3. Coadministration of hyaluronidase increases the antitumor efficacy of Ad5 (OV-5) and fiber-chimeric oncolytic viruses (OV-5T35H) in a spontaneous metastatic melanoma (A375-mln1.luc) model. Nude mice (n = 16) bearing s.c. A375-mln1.luc tumors (100 mm³) received 4 injections of either PBS, rHuPH20 enzyme (50 U per mouse per injection), virus (OV-5 or OV-5T35H; 1 × 10¹⁰ vp), or coinjection of OV-5 or OV-5T35H plus rHuPH20 once every other day. Tumor progression (A) and survival (B) were monitored over time. A statistically significant delay in tumor progression was observed between the vector-treated groups compared with the Ad5 parental or Ad5 parental plus enzyme – treated groups (OV-5T35H+rHuPH20 versus OV-5 or OV-5 +rHuPH20, P < 0.05).


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other day, of either PBS, rHuPH20 (50 or 250 U per injection), OV-5T35H virus (1 × 10^{10} vp per injection), or OV-5T35H1 (1 × 10^{10} vp per injection) with rHuPH20 (50 U per injection). Mice were euthanized on days 8 and 13, and the presence of luciferase-positive tumor cells in LN and lungs was determined by measuring the level of luciferase activity in pooled organs after direct injection with luciferin (11).

Total photon counts observed in LN and lungs at both time points in the groups treated with oncolytic virus were greatly reduced when compared with the organs from tumor-bearing animals treated with either PBS or hyaluronidase alone (Fig. 5A-C), indicating that the metastatic spread of tumor cells was reduced in virus-treated animals. At day 8, there was a ~10-fold reduction in photon counts in LN and lungs in the groups treated with either OV-5T35H or OV-5T35H plus rHuPH20 compared with the groups treated with either PBS or enzyme alone. However, there was no difference in photon counts between the groups treated with OV-5T35H or the combination of OV-5T35H with enzyme at this time point.

At day 13, there was no difference in photon counts in LN and lungs of animals injected with PBS alone or treated with either 50 or 250 U of enzyme. Interestingly, there was a 50-fold reduction in photon counts in LN and lungs of mice treated with the chimeric virus plus rHuPH20 compared with those treated with the chimeric virus alone. These data suggested that the enzyme either alone or in combination with OV-5T35H did not increase the metastatic potential of the highly metastatic A375-mln1.luc tumor cells.

**Discussion**

The relatively limited ability of Ad5-based oncolytic viruses to transduce a broad range of tumor cells and to spread efficiently throughout solid tumors may hamper their effectiveness in the clinic. A fiber knob replacement strategy, generating Ad5/35 oncolytic viruses with chimeric fiber proteins, has previously been shown to improve the transduction efficiency of such viruses significantly (7, 11). However, there is still a need for approaches that improve spread of these viruses throughout tumors.

In a previous study, engineering the matrix-degrading protein relaxin into an Ad5/35 fiber-chimeric virus improved both transduction efficiency and virus spread within tumors (11). To increase ECM degradation during virus transduction without modifications to the virus genome, a different approach to improve viral spread was used in this study. Ad5-based or Ad5/35 fiber chimeric viruses were coinjected intratumorally with the rHuPH20, which has been shown to degrade hyaluronan transiently in vivo (13, 16).

Although hyaluronidase and relaxin (11, 32) both degrade components within the ECM thereby increasing virus spread, hyaluronidase coinjection has the advantage of not requiring

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**Fig. 4.** Coadministration of rHuPH20 enzyme increases virus spread in A375-mln1.luc tumors. Nude mice (n = 16) bearing s.c. A375-mln1.luc tumors at a size of (100 mm^3) received four intratumoral injections of either PBS, rHuPH20 enzyme (50 U per injection), virus (OV-5 or OV-5T35H; 1 × 10^{10} vp), or coinjection of OV-5 or OV-5T35H plus rHuPH20 once every other day. Ten days after the initial viral injection, tumors were harvested (n = 3) and (A) virus distribution was evaluated by determining the number of hexon-positive cells within tumors by immunohistochemistry on paraffin-embedded tumor tissue (×10). B, the amount of infectious virus present in tumors was determined by titrating tumor lysates on A51-2A cells.
Fig. 5. Evaluation of the effect of rHuPH20 enzyme on metastasis of A375-mln1 luc tumor cells. Nude mice (n = 10) bearing s.c. A375-mln1 luc tumors (100 mm³) received a total of four intratumoral injections (50 µL) of PBS, rHuPH20 enzyme (50 or 250 U per injection), OV-5T35H virus (1 x 10¹⁰ vp per injection), or coinjection of OV-5T35H plus rHuPH20 once every other day. The level of metastasis was evaluated on (A) day 8 and (B) day 13 postinjection in isolated LN and lungs by injecting them with luciferin to determine photon counts using the IVIS® Xenogen system. The combined total average photon counts from LN and lungs is shown (n = 5). C, actual IVIS images of LN and lung on Day 13.
manipulation of the adenovirus genome and can therefore be added to different viruses. In addition, by knowing the half-life of hyaluronidase, one has control over the amount of enzyme delivered to the tumor. In contrast, viruses expressing relaxin will produce relaxin as long as they replicate within the tumor cells, which might differ between tumors.

The current data show that co-injection of hyaluronidase at a concentration of 50 U with an E1-deleted Ad5/35 fiber chimeric virus significantly improves viral transduction of PC-3.luc tumors. Fifty enzyme units was selected from pilot experiments showing that tumors injected with less (10 U; data not shown) or more enzyme (200 or 800 U) showed fewer GFP-positive cells than tumors that were injected with 50 U of enzyme. Ten enzyme units might be too little to break down the ECM within tumors well enough to allow effective virus spread; however, the reason for why 200 and 800 U of enzyme did less well than 50 U is currently not known. One potential explanation could be that higher concentrations of enzyme, which is premixed with the virus before injection into the tumor, are slightly toxic to the virus; however, in vitro studies did not support this hypothesis. Due to lack of access to reagents that allow quantification of hyaluronic acid content within tumors, it was impossible to look at effect of various hyaluronidase concentrations on overall hyaluronic acid content within the tumor models used in this study. Nevertheless, based on the optimal virus spread observed with 50 U of enzyme, this concentration was selected for further studies.

The observed increase in virus transduction as a result of hyaluronidase treatment correlates with improved antitumor activity when oncolytic adenoviruses are co-injected with hyaluronidase in murine tumor models. Combination treatment with both chimeric Ad5/35 virus and enzyme improves the oncolytic potency of the virus and significantly increases survival of tumor-bearing mice. Importantly, this combination also effectively controls the growth of tumors that are two times as large (250 mm³) as those typically used in these models and significantly prolongs the survival of the treated animals emphasizing the overall potency of this combination.

To determine if hyaluronidase co-injection has a different effect on the potency of viruses that transduce tumors with different efficiencies, the parental OV-5 and fiber-chimeric oncolytic virus OV-5T35H were tested with and without enzyme in the highly metastatic A375-mln1.luc melanoma tumor model. In this model, the combination of hyaluronidase and either virus resulted in improved antitumor efficacy and prolonged survival of tumor-bearing mice compared with monotherapy with either virus. It is notable that co-injecting hyaluronidase with the chimeric virus increases survival significantly, and the MST is not attained by day 65, with 67% of animals still alive at this time point. Interestingly, the increase in viral titer observed in the tumors treated with the combination of OV-5T35H and enzyme was only ~2-3-fold compared with tumors treated with OV-5T35H alone, whereas there was a ~10-fold increase in virus titers in tumors treated with OV-5 with enzyme compared with OV-5 alone. Apparently, the enzyme enhanced the spread of the potent chimeric virus to a lesser extent than the spread of the Ad5-based parental virus, perhaps due to the higher initial infectivity of the chimeric virus in the A375.ml1.luc tumor model. Nevertheless, the addition of rHuPH20 treatment to the chimeric virus resulted in a pronounced survival advantage compared with treatment with the chimeric virus alone. In the context of the relatively poorly transducing OV-5 virus, coinjection of rHuPH20 significantly improved virus spread that also was reflected in a significant survival advantage. Overall, more infectious virus was observed in tumors treated with OV-5T35H compared with OV-5, indicating a greater level of intratumoral virus replication and spread of the chimeric virus.

In summary, the increase in virus potency observed in animals treated with both enzyme and virus correlates with an improved spread of virus within the tumors, as shown by the presence of increased numbers of hexon-positive cells, which in turn correlates with an increase in the amount of infectious virus present within these tumors. Because the majority of human cancers express the CD46 receptor of the Ad5/35 chimera more consistently and at higher levels than the coxsackievirus-adenovirus receptor of the Ad5 virus, these findings suggest that the combination of hyaluronidase and chimeric virus may be a more optimal therapy.

It is important to consider the possibility that the enzyme, which degrades the ECM, could increase metastatic spread of tumor cells not killed by the oncolytic virus. To address this concern, highly metastatic A375-mln1.luc tumors were injected with the chimeric virus with or without hyaluronidase, or with enzyme alone, and the animals were screened for the presence of tumor cells in LN and lungs at two time points. Metastasis to LN and lung at day 13 was greatly reduced in animals treated with chimeric virus and hyaluronidase compared with animals treated with virus or enzyme alone. These results are most likely due to the reduced growth of primary tumors treated with the combination compared with tumors treated with either virus or enzyme alone, because metastatic spread is believed to be related to the size of the primary tumor in this model. Hyaluronidase by itself, even at a 5-fold higher dose, does not increase the metastatic spread of the A375-mln1.luc tumors. This observation is in agreement with a report from Bookbinder et al. (17), demonstrating that hyaluronidase enhances the infusion rates and penetration of molecules up to 200 nm in diameter, a size range that would allow adenoviruses but not tumor cells to penetrate throughout tissues. Another explanation could be that the better spread of virus within tumors treated with the enzyme resulted in more effective killing of the tumor cells by the increased amounts of virus, and that the virus-mediated killing of tumor cells preceded the escape of the cells from the primary tumor mass into the vascular system.

In conclusion, the data presented here show that combination treatment of solid tumors with an oncolytic virus in which the Ad5 fiber knob has been replaced with the Ad35 knob with human hyaluronidase significantly improves transduction efficiency and spread of virus through the tumor, resulting in prolonged survival of the tumor-bearing animals without increasing the metastatic potential. Thus, this combination of oncolytic adenovirus and hyaluronidase may offer a novel and promising therapy for the treatment of patients with cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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