Heat Shock and Heat Shock Protein 70i Enhance the Oncolytic Effect of Replicative Adenovirus

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Abstract

Replication-competent viruses are currently being evaluated for their cancer cell-killing properties. These vectors are designed to induce tumor regression after selective viral propagation within the tumor. However, replication-competent viruses have not resulted heretofore in complete tumor eradication in the clinical setting. Recently, heat shock has been reported to partially alleviate replication restriction on an avian adenovirus (Ad) in a human lung cancer cell line. Therefore, we hypothesized that heat shock and overexpression of heat shock protein (hsp) would support the oncolytic effect of a replication-competent human Ad. To this end, we tested the oncolytic and burst kinetics of a replication-competent Ad after exposure to heat shock or to inducible hsp 70 overexpression by a replication-deficient Ad (Adhsp 70i). Heat-shock resulted in augmentation of Ad burst and oncolysis while decreasing total intracellular Ad DNA. Overexpression of hsp 70i also enhanced Ad-mediated oncolysis but did not decrease intracellular Ad DNA levels. We conclude that heat shock and Adhsp 70i enhance the Ad cell-killing potential via distinct mechanisms. A potential therapeutic implication would be the use of local hyperthermia to augment oncolysis by increasing the burst of replication-competent Ad. The role of hsp in Ad-mediated oncolysis should be additionally explored.

Introduction

Because malignant tumors are of a highly complex nature, a cure will probably result only from complete eradication of all of the tumor cells. Current modalities for cancer therapy are not selective and may have severe adverse effects. Therefore, cancer gene therapy has emerged as an alternative and promising new modality after the recent advances in understanding cancer biology. Despite the initial encouraging preclinical studies using replication-incompetent viral vectors, these agents failed to result in significant benefit in the clinical setting.

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Advances in Brief

Replicative Adenovirus

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Because malignant tumors are of a highly complex nature, a cure will probably result only from complete eradication of all of the tumor cells. Current modalities for cancer therapy are not selective and may have severe adverse effects. Therefore, cancer gene therapy has emerged as an alternative and promising new modality after the recent advances in understanding cancer biology. Despite the initial encouraging preclinical studies using replication-incompetent viral vectors, these agents failed to result in significant benefit in the clinical setting. A natural evolution of this course was the introduction of RCVs for cancer therapy (1), where selective viral self-perpetuation is suggested to eradicate or decrease tumor mass and augment the host immune response to tumor antigens. Despite the use of other vectors, human Ads are the leading agents for cancer gene therapy. Whereas the life cycle of Ad has not been completely elucidated, it is clear that it requires redirection of the host cellular biochemical machinery by viral gene products. Viral replication is dependent on the host cell for genome replication, protein synthesis, and virion assembly. In this regard, hsps may play an important role in the Ad life cycle (2). It has been demonstrated for the avian Ad CELO that induction of hsp 40 and 70 allowed production of viral proteins and virions. Importantly, the restriction on a mutant CELO Ad replication was alleviated by both heat shock and hsp overexpression. Previous supportive data regarding the role of hsp in Ad infection also include correlation of hsp levels with permissiveness of human cells to Ad infection (3) and selective hsp 70 mRNA transport occurring late in Ad infection (4).

Consequently, we hypothesized that heat shock and hsp overexpression would alter Ad life cycle in cancer cells to enhance oncolysis. To test our hypothesis, we heat-shocked lung cancer cell lines after Ad infection and demonstrated augmentation of Ad burst and oncolysis. Next, we overexpressed the inducible hsp 70 with a replication-incompetent Ad vector and have shown that it also enhances Ad-mediated oncolysis. However, Ad burst patterns differ after cellular exposure to heat shock or hsp 70i overexpression.

Materials and Methods

Cell Lines and Viruses. The lung cancer cell lines A549 and H460 were obtained from the American Type Culture Collection (Manassas, VA), and H157 was a gift of Koichi Takayama (Princeton University, Princeton, NJ). An E3-deleted, replication-competent human Ad5 expressing luciferase (Ad5luc3) was used in this study as a replication-competent equivalent of wild-type human Ad5. An E1-deleted, replication-incompetent Ad5 expressing luciferase (Ad5luc1) was used as a control virus. Ad338 is a replication-competent, E1B-55,000-deleted Ad5, kindly provided by Tom Shenk (5). Adhsp 70i is an E1-deleted Ad expressing the inducible hsp 70 under the transcriptional control of the human CMV enhancer/promoter and was obtained from Ruben Mestril (University of California, San Diego, La Jolla, CA) (6).

Infections, Heat Shock, and Cell Viability Assays. A549, H460, and H157 cells were grown in a Ham's F12K medium with 2 m M -glutamine, supplemented by 10% fetal bovine serum at 37 C°. For the heat shock experiments, plates were incubated in a Forma Scientific incubator (Marietta, OH) at 37 C° and heat shocked daily at 42.5 C° for various periods in a distinct designated incubator. Temperature was validated with two different thermometers. Plates were simultaneously stained with crystal violet once an advanced CPE was identified for either of the groups. For the experiments with Adhsp 70i, A549 cells were grown as before. A549 cells were infected with Adhsp 70i at an MOI of 10 for 1 h in a serum-free medium. Ad5luc1 served as a control replication-incompetent Ad. After 30 h, we infected with the replication-competent Ad5luc3 at an MOI of 5 under the same conditions as above. Medium was sampled daily for E1a gene copy numbers, and all of the plates were stained simultaneously with crystal violet once an advanced CPE was identified for either of the groups. Quantitative cell killing evaluation of heat

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3 The abbreviations used are: RCV, replication-competent virus; Ad, adenovirus; hsp, heat shock protein; MOI, multiplicity of infection; CPE, cytopathic effect; HPV, human papillomavirus; Tag, T antigen.

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shocked cells was performed with the MTS assay using the CellTiter 96 AQ Cell Proliferation assay (Promega, WI), modified for 24-well plates. Cells were infected at an MOI of 1 and evaluated at different time points as depicted in Fig. 1B. MTS reagent (80 μl) was added to each well before determination of OD by 490 nm absorbance of formazan measured directly from the 24-well plates.

TaqMan PCR Assay. E1a copy numbers were determined for each medium sample obtained in triplicates as of the first day after infection. Genomic DNA was isolated and cleaned using a Qiagen Tissue kit (Qiagen, Santa Clara, CA) according to the instructions of the manufacturer. Concentration of purified DNA was determined by A260. The design of TaqMan primers and probe was as follows: the forward primer, reverse primer, and 6-FAM labeled probe to amplify the E1a gene were designed by the Primer Express 1.0 software (Perkin-Elmer, Foster City, CA) and are available on request.

With optimized concentration of primers and probe, the components of real time PCR mixture were designed to result in a master mix with a final volume of 10 μl/reaction containing IX Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 100 nM forward primer, 100 nM reverse primer, 1 nM probe, and 0.025% BSA. For the assay, 1 μl of extracted DNA sample was added to 10 μl of PCR mixture in each reaction capillary. A no-template control received 10 μl of reaction mixture with 1 μl of water. All of the capillaries were then sealed and centrifuged using LC Carousel Centrifuge (Roche Molecular Biochemicals, Indianapolis, IN) to facilitate mixing. All of the PCR was carried out using a LightCycler System (Roche Molecular Biochemicals). The thermal cycling conditions were 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C.

Results

Heat Shock Augments the Oncolytic Effect of Replicative Ad. To evaluate the hypothesis that the oncolytic potency of replicating Ad is enhanced by heat shock, we first examined the effect of hyperthermia on Ad5luc3-mediated oncolysis in A549 cells. Heat shock induces apoptotic cancer cell killing at temperatures ~42°C, whereas at higher temperatures cell death is necrotic (7). Another important result of heat shock is the induction of a variety of hsp (8). Whereas the maximal rate of hsp synthesis occurs 3–5 h after heat shock, hsp levels may remain elevated for days after hyperthermic exposure. To evaluate the effect of heat shock on Ad-mediated oncolysis, we heat shocked A549 cells as of the first day after infection for either 1 or 2 h daily. For these studies, Ad5luc3 was selected, because it is an E3-deleted replicating virus expressing luciferase. Therefore, we eliminated the potential lytic role of the Ad death protein and normalized for transgene expression with the control replication-incompetent virus, Ad5luc1. Consequently, the variable determining the outcome of infection was Ad5 life cycle within the tumor cells. To normalize for heat-induced toxicity, in each 12-well plate a triplicate was mock-infected, thereby subject only to the effect of the variable heat shock periods. We selected 42.5°C as an optimal temperature for heat shock induction, because Ad5 is partially inactivated at 43–44°C, and because temperature below 42°C may yield less than optimal hsp induction (8). Longer periods of heat exposure were toxic to the cells, whereas repetitive exposures up to 2–4 h/day in all, were tolerable. To carefully characterize the heat shock effect on viral burst and oncolysis, we used low MOIs, from 0.01 to 1 viral plaque-forming unit/cell.

The replication-incompetent Ad5luc1 did not induce any CPE for the MOIs tested, with or without heat shock, for as long as 14 days after infection. However, for the replicating Ad5luc3, CPE was evident as of 4–5 days after infection. The earliest CPE was documented for cells infected with an MOI of 1 and exposed to daily heat shock (Fig. 1A). Cells infected with same MOI that were not heat shocked displayed an equivalent degree of CPE at least 24 h later, whereas cells that were heat shocked 2 h daily had a higher degree of CPE than cells heat shocked for 1 h daily. Of note, the degree of CPE obtained for cells heat shocked for 2 h daily after infection with 0.01 or 0.1 MOI was equivalent at that time point to that observed for non-heated shocked cells infected with an MOI 1–2 order of magnitude higher. These findings are in agreement with both the characteristics of time-dependent heat shock induced apoptosis (9) and time-dependent hsp 70 induction (10), and indicate that the length of the heat shock affects Ad-mediated oncolysis. Because mock-infected cells exposed to identical heat shock periods grew as well as the nonheated cells (Fig. 1A, left lanes), the conditions of heat shock we used did not induce cell toxicity independently but rather contributed synergistically to Ad5-mediated oncolysis. Thus, controlled hyperthermia supports Ad5-mediated oncolysis.

To analyze quantitatively the effect of heat shock on the in vitro Ad oncolysis, an MTS cell viability assay was performed (Fig. 1B). The infection protocol for the MTS assay was identical to the crystal violet assay. The cell killing pattern induced by heat shock suggests different kinetics for the heat shocked groups where cell viability rapidly deteriorates, whereas the rate of the non-heated cell killing is moderate. In comparison to the heat shocked group, the nonheated cells had a late and relatively slower rate of lysis.

Statistical Analysis. Data were initially tested for normality by the Shapiro-Wilk test. All of the abnormal tests were additionally tested for significance by the Wilcoxon-scores test. Results are expressed as a mean of at least three samples for E1a copy number assay or six samples for the MTS assay. Results were considered statistically significant for a P < 0.05.
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These experiments confirmed the enhanced effect of controlled heat shock on Ad5 oncolysis and were also documented for two other lung cancer cell lines, H460 and H157. As well, other replication-competent recombinant Ad5 viruses, including the attenuated E1B-55,000-deleted Ad 338, had a higher oncolytic potency in combination with heat shock (data not shown). Thus, heat shock-enhanced Ad oncolysis may be a general feature of replication-competent Ad.

Heat Shock-enhanced Adenoviral Oncolysis Is Mediated by Facilitated Viral Burst. To investigate the mechanism of heat shock-enhanced Ad5 oncolysis, we evaluated the patterns of Ad5 release from A549 cells. Ad viruses lyse cells after their replication and release from the cell. Therefore, we reasoned that determination of the kinetics of Ad5 release from the cells would elucidate the mechanism of heat shock-enhanced oncolysis. To this end, we quantitatively evaluated Ad DNA in the medium and cellular fractions by determining Ad5 E1a gene copy numbers with the TaqMan assay, as used previously to quantitate Ad DNA (11). Analysis of Ad DNA in the medium clearly indicates that heat shock results in earlier and higher viral release to the medium (Fig. 2A). The heat-induced Ad burst is in accordance with the augmented cell killing effect of heat shock on Ad oncolysis, suggesting that it represents the mechanism of heat-enhanced Ad oncolysis. Because either augmented viral replication or earlier cell death may be the primary event resulting in earlier Ad burst, we additionally analyzed the number of intracellular Ad E1a gene copies in various time points after infection as an index of Ad replication (Fig. 2B). The nonheated cells produced continuously rising numbers of Ad DNA copies, indicating ongoing Ad replication and infection. In contrast, in the heat shocked cells, the total Ad5 gene copy number leveled off 2–3 days after infection.

Therefore, augmented Ad replication is not the primary mechanism of heat-enhanced oncolysis. Rather, primary heat-induced Ad burst may account for the enhanced cell killing, the rising concentration of Ad DNA in the medium, and for the constantly low intracellular Ad DNA levels. Heat shock may result in premature death of infected cells, rendering Ad progeny available for more infections. Alternatively, heat shock may affect directly the life cycle of Ad. Whereas these two scenarios need additional studies, our data clearly show that for the in vitro cell killing assays we used, hyperthermia alone is insufficient to achieve significant cancer cell killing, whereas it dramatically alters the natural course of cellular infection with a replicating Ad. Of note, we also demonstrated induction of Ad5 burst by irradiation (data not shown), but this phenomenon requires additional studies. Taken together, our data indicate that heat shock efficiently induces in vitro a shift of Ad5 from the cellular component to the medium, representing earlier Ad burst and cell death.

Adhsp 70i Supports Adenoviral-mediated Oncolysis. After the determination of the synergistic effect of heat shock on Ad5 burst and oncolysis, we turned to examine the direct effect of hsp 70i, the major cellular stress protein, on Ad-mediated cell killing. We hypothesized that hsp 70i overexpression by the replication-deficient Adhsp 70i vector would augment the oncolytic effect of a replicating Ad5. To this end, we preinfected A549 cells at an MOI of 10 with the replication-incompetent viruses, either Adhsp 70i or the control Ad5luc1. Later (30 h), cells were infected with the replication-competent Ad5luc3 at an MOI of 5.

A simultaneous coinfection of a replicating virus and either one of these nonreplicating viruses resulted in rapid oncolysis at both groups, probably caused by E1 trans-complementation of the nonreplicating viruses. Therefore, to decrease the trans-complementation and delineate the isolated effect of hsp 70i overexpression on Ad5 replication and oncolysis, the time interval from infection with the replication-deficient Ad was essential. As early as 36 h after infection with Ad5luc3, we could notice typical CPE for the Adhsp 70i preinfected group. At this stage, no CPE was apparent for the cells infected with either the replicating virus alone or in combination with Ad5luc1 (Fig. 3A). Therefore, hsp 70i overexpression by Adhsp 70i is synergistic to the cell killing effect of replicative Ad. Next, we sought to evaluate the effect of Adhsp 70i on the kinetics of Ad5luc3. Infection with Adhsp 70i or Ad5luc1 was performed exactly as for the cell killing assay. We simultaneously sampled daily both the medium, and from a different set of plates, the cellular fraction, for E1a copy number measurement with the quantitative TaqMan PCR. When compared with Ad5luc3 in combination with the control Ad5luc1, Adhsp 70i induced an earlier and higher Ad5luc3 burst rate, as determined by the quantitative determination of Ad5 gene copies in the medium (Fig. 3B). However, this difference did not reach statistical significance. When evaluating the cellular fraction of Ad5luc3 E1a gene copies in cells preinfected with Adhsp 70i, we found that viral DNA was more abundant in comparison to the cells preinfected with Ad5luc1 (Fig. 3C). Although this difference was also not statistically significant, it is clearly distinct from the inhibition of cellular Ad DNA accumulation induced by heat shock. These data may indicate that Adhsp 70i confers a selective advantage for Ad5-mediated cell killing, but unlike heat shock, not via an isolated effect on Ad5 burst.

Discussion

Cancer gene therapy is currently limited by the inadequacy of vectors to completely eliminate the malignant clone. Therefore, RCVs have been proposed to lyse cells and propagate throughout the tumor mass. However, clinical trials with local injection of RCV for head and neck cancer have shown that whereas an acceptable safety level was achieved, oncolytic efficacy clearly requires improved potency (12). In this regard, we have investigated the hypothesis that hyperthermia and hsp 70i overexpression would support Ad5-mediated
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We investigated whether heat shock (42 °C) could augment adenovirus (Ad) burst and cell death in a canine distemper virus (CDV)-infected canine fibroblast cell line. Ad5luc1 was infected at an MOI of 2, and Adhsp70i at an MOI of 5. Medium was sampled daily in triplicates as of the first day after infection. A549 cells were infected exactly as in (A) with Ad5luc1 ( ), and 30 h later, cells were infected with the replication-competent Ad ( ). A549 cells were infected exactly as in (B) with Ad5luc3 ( ), or Adhsp70i ( ). Later (30 h), cells were stained with crystal violet after observation of advanced CPE. Mock represents mock-infected cells. A, kinetics of Ad burst after hsp 70i overexpression. A549 cells were infected exactly as in A with Ad5luc1 ( ) or Ad5hsp70i ( ). Later (30 h), all of the cells were infected with the replication-competent Ad5luc3 at an MOI of 5. Medium was sampled daily in triplicates as of the first day after infection with Ad5luc3 for future determination of E1a gene copy number. B, analysis of intracellular Ad DNA replication by determination of E1a gene copy number. A549 cells were infected exactly as in A with Ad5luc3 ( ) or Adhsp70i ( ). On the indicated time periods after infection with Ad5luc3, cells were harvested for total DNA extraction.

To address this hypothesis, we first evaluated the cell killing effect and the kinetics of Ad5 burst after a daily heat shock. We have shown that a controlled heat shock augments Ad5 burst, thereby increasing its oncolytic potency. Because the combination of heat shock and a replication-deficient Ad5 did not increase cell killing, this effect of heat shock is unique to replication-competent Ad. Finally, we demonstrated that Ad5 burst is supported by Adhsp70i, albeit via an alternate mechanism that is not restricted to viral burst. These findings are reminiscent of the paradoxical enhancement of viral CPE by the canine distemper virus after induction of the stress response (13). Our findings also agree with clinical data, where isolated hyperthermic strategies (14) have shown to increase the available hyperthermic strategies (14). In this regard, our studies showed that hsp 70i overexpression also resulted in a higher degree of cell killing. However, unlike heat shock, Ad replicated well in the cellular fraction after hsp 70i overexpression. We could not demonstrate a statistically significant increase in Ad replication after hsp 70i expression, but DNA levels of the replication-competent Ad were higher both at the cellular level and in the medium. Because cellular Ad DNA levels were time-dependent after infection, it seems that isolated viral burst is not the primary mechanism responsible for Adhsp 70i-enhanced oncolysis. Possible mechanisms for this finding may stem from the timely hsp 70 induction by Ad, considered important for intracellular Ad life cycle. Specifically, both Ad5 infection and heat shock induce stabilization of the filamentous actin network (17), and hsp 70 both promotes import of viral particles and colocalizes in the nucleus with Ad E1a (18). Furthermore, specific induction of hsp 70 by E1A during a lytic adenoviral infection is well characterized (3, 19). Viral DNA replication may also depend on hsp, as was documented for bacterial DnaK and DnaJ that are essential for bacteriophage DNA replication (20). A role of their human homologues hsp 70 and hsp 40 for small DNA tumor viruses has been described recently.

Specifically, because the J domain of hsp 40 usually stimulates the ATPase activity of hsp 70, it binds to and multimerizes the HPV E1 protein in the process of replication initiation (21) and is necessary for efficient SV40 DNA replication (22). hsp also play a role in binding denatured nuclear proteins and preventing their aggregation and disruption of nuclear matrix-dependent DNA replication and transcription, hsp 70 stimulates viral transcription in cells infected with measles virus and canine distemper virus (23). Whereas there is no direct evidence for hsp involvement in transcription of Ad genes, chaperones were reported to be involved in transcriptional regulation (24). Of note, despite the general host cell protein shut-off during the late phase of Ad infection, selective translation of hsp mRNA is maintained, thereby suggesting that synchronized expression of hsp in Ad-infected cells may confer a selective advantage for the viral life cycle (25). Ribosomal shunting, the bypass of large mRNA segments before initiating translation at a downstream AUG, is directed by the Ad tripartite leader, and provides preferential translation to late Ad mRNA (26). During heat shock, the shunting mechanism is used exclusively by the hsp 70 mRNA. Elements within hsp 70 mRNA are related to those found in the Ad tripartite leader mRNA and share a unique property of promoting translation during the late phase of the Ad infectious cycle. Interestingly, elevated temperature can alleviate the defect of the Ad E1b Mtr 55,000 mutant in mRNA transport, and the late gene expression and progeny production (27).

hsp 70 may also be involved at the late stages of Ad assembly, because its maximal synthesis concurs with the log phase of Ad structural assembly (28). This may be essential for virion production, because only 10% of structural Ad proteins synthesized are eventually assembled into virions (28). hsp 70 protein has been found to be associated with Ad capsid, to colocalize with the Ad particles in the nucleus (3), and to associate with hexon, the major Ad capsid protein (29), and with the fiber discharge from the cell correlates with cell killing, and both are enhanced by heat shock.

One mechanism to explain this observation may involve the induction of apoptosis by heat. While by itself insufficient to result in significant cell killing in our study, heat shock can efficiently induce apoptosis (7, 15). Importantly, induction of apoptosis by exposure to 42 °C in combination with chemotherapy has been reported to be more efficient than by hyperthermia of 44 °C (16). We speculate that apoptosis may affect the release of viral progeny after completion of the viral life cycle. This hypothesis is currently being studied. Another attractive mechanism for heat shock-enhanced oncolysis would be the induction of hsp. In this regard, our studies showed that hsp 70i overexpression also resulted in a higher degree of cell killing. However, unlike heat shock, Ad replicated well in the cellular fraction after hsp 70i overexpression. We could not demonstrate a statistically significant increase in Ad replication after hsp 70i expression, but DNA levels of the replication-competent Ad were higher both at the cellular level and in the medium. Because cellular Ad DNA levels were time-dependent after infection, it seems that isolated viral burst is not the primary mechanism responsible for Adhsp 70i-enhanced oncolysis. Possible mechanisms for this finding may stem from the timely hsp 70 induction by Ad, considered important for intracellular Ad life cycle. Specifically, both Ad5 infection and heat shock induce stabilization of the filamentous actin network (17), and hsp 70 both promotes import of viral particles and colocalizes in the nucleus with Ad E1a (18). Furthermore, specific induction of hsp 70 by E1A during a lytic adenoviral infection is well characterized (3, 19). Viral DNA replication may also depend on hsp, as was documented for bacterial DnaK and DnaJ that are essential for bacteriophage DNA replication (20). A role of their human homologues hsp 70 and hsp 40 for small DNA tumor viruses has been described recently.
protein (28). The latter is the only Ad structural protein that requires glycosylation, where hsp 70 may direct its transport from the endoplasmic reticulum to the nucleus for virion assembly (30). The monomers of Ad5 hexon and fiber are insoluble and may need the cellular chaperone machinery to mediate, and perhaps regulate, their assembly into oligomeric complexes.

Another emerging pathway that may be related to Ad redirection of cellular machinery in the context of hsp is cellular transformation. For other small DNA tumor viruses, hsps have been found to play a major role in displacement of E2F from the Rb protein family. The SV40 large Tag requires an NH2-terminal domain for cell transformation (21). This domain exhibits functional and sequence homology with the J domain of the bacterial DnaJ protein and the hsp 40 family. The NH2-terminal domain of Tag interacts with hsp 70 and stimulates hsp 70-mediated disassembly of the Rh-E2F complex. Unlike SV40 and polyomavirus that execute an endogenous J domain in their Tag, HPV uses the host hsp 40. Because the LXCXE motif, mediating the binding to the pocket domain of the Rh protein family, is embodied in the Ad E1a and HPV E7 genes, it would be tempting to speculate that Ad may use the J domain of cellular hsp 40 to drive hsp 70-mediated Rh-E2F complex disassembly. Indeed, high levels of hsp 70 have been linked to early Ad gene expression in the absence of E1A (3).

In conclusion, this study demonstrates the induction of Ad5 burst and potentiation of its oncolytic effect by heat shock. Adhsp 70i infection also enhanced Ad-induced oncolysis but via a distinct, yet undefined mechanism.

Future implications of these findings may involve the use of hyperthermia to enhance the local oncolytic effect of Ad vectors in vivo, as well as additional exploration of a potential role of hsp in Ad-mediated oncolysis.

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