Endocr (2010) 37:430–439
DOI 10.1007/s12020-010-9333-5

ORIGINAL ARTICLE

Anti-tumor effects of adenovirus containing human growth hormone sequences in a mouse model of human ovarian cancer

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Received: 30 January 2009 / Accepted: 12 March 2010 / Published online: 20 April 2010

Abstract Women with ovarian cancer have a low survival rate and develop resistance to chemotherapy, so new approaches to treatment are needed. We unexpectedly found administration of a replication-deficient adenovirus containing human growth hormone sequences (AdXGH) was beneficial in a mouse model of human ovarian cancer. Intraperitoneal injections of AdXGH prolonged median survival from a mean of 31 ± 1.2 to 40 ± 1.4 days in immunodeficient SCID mice given SKOV3.ip1 human ovarian cancer cells in the peritoneal cavity. Adenovirus containing human prolactin or del32-71growth hormone sequences had no effect. Repeated injection of growth hormone or implantation of tablets with sustained growth hormone release did not increase survival. Control mice had overlapping tumors throughout the peritoneal cavity and liver and frequent lung metastases 24 days after tumor cell injection. Mice that received two injections of AdXGH had no lung metastases. Mice that received four injections had no lung or liver metastases and peritoneal fibrosis. They did not survive longer than mice that received two injections, but they had enlarged livers with hepatocellular changes, indicating that a limitation of increasing the dose is liver toxicity.

Keywords Ovarian cancer · Growth hormone · Adenovirus · Fibrosis

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Introduction

Ovarian cancer is the fifth leading cause of cancer among women. Ovarian cancer is usually diagnosed at late stages and most patients have a 5 year survival rate that is <30%. This low survival rate has not changed markedly in decades [1, 2]. Most patients respond to chemotherapy initially, but later develop resistance, resulting in low rates of permanent remission. New approaches to treating late stage ovarian cancer are needed.

We used a mouse model of human ovarian cancer to test a possible treatment—the model is SKOV3.ip1 cells, human ovarian cancer cells [3], injected into the peritoneal cavity of severe combined immunodeficiency (SCID) mice. SCID mice tolerate implantation of human cells because these mice carry a mutation that impairs the differentiation of B and T cells into functioning cells [4]. SKOV3 cells form tumors in these mice that resemble ovarian cancer in women: (1) carcinatomatosis is initially confined to the peritoneal cavity and there may be metastases in the lungs at later stages. (2) Progression of the disease includes ascites formation. (3) The morphology of the tumors closely resembles peritoneal metastases from poorly differentiated stage III ovarian cancer [5].

We unexpectedly found that administration of replication-deficient adenovirus containing human growth hormone sequences was beneficial in this mouse model. We

were testing whether forcing cells to express a protein in the secretory pathway that does not fold properly would reduce progression of ovarian cancer. Many membrane and secretory proteins are synthesized in the endoplasmic reticulum; those that do not fold properly may accumulate there, since proteins must be in appropriate conformations to be transported efficiently from the endoplasmic reticulum [6]. Rapidly growing cells, such as cancer cells, would be expected to need a fully functioning secretory pathway. Human del32-71 growth hormone, a protein that cannot fold properly, is rapidly degraded in cells that secrete large amounts of protein, but in other cells it accumulates in the endoplasmic reticulum and causes the Golgi complex to fragment [7, 8]. We tested whether cancer cells in animals would not grow as well if made to express this protein, and used a model for human ovarian cancer since there has been so little progress with this type of cancer. We used replication-deficient adenovirus to force cells to express del32-71 growth hormone (AdXdel32-71GH) or, as a control, to express human growth hormone (AdXGH). Human growth hormone does fold and is properly secreted in many cell types [7]. We found that treatment with AdXGH, intended to be the control, prolonged survival and reduced tumor burden.

Results

AdXGH administration prolongs survival

Mice were injected with human ovarian cancer cells, SKOV3.ip1, in the peritoneal cavity and then injected with AdXGH or AdXdel32-71GH 4 and 8 days after tumor cell injection (Fig. 1). Control mice treated with phosphate-buffered saline (PBS) had a median survival of 29 days after tumor cell injection and those treated with AdXdel32-71GH, 29.5 days. Unexpectedly, mice treated with AdXGH had a median survival time of 42 days ($P < 0.0003$ compared with controls) and two mice in this group lived much longer (Fig. 1).

Ovarian cancer is usually detected and treated in women at late stages [1, 2], so we tested whether AdXGH treatment is effective when the SKOV3.ip1 cells have had longer to establish tumors. Mice that were injected with AdXGH at 10 and 14 days after tumor cell injection had a median survival of 44 days and controls, 37 days ($P < 0.016$), so injections are beneficial at later times (Fig. 2).

We performed a total of nine experiments using SCID mice aged 9–11 weeks, and the ability to prolong survival with AdXGH was reproducible in all these experiments. The mean of the median survival times for these nine experiments was 40 ± 1.4 days in mice treated with AdXGH, significantly different than that of controls, 31 ± 1.2 days ($P < 0.0002$). The difference in median survival between control and AdXGH-treated mice ranged from 5 to 13 days in the nine experiments. Other laboratories that have used the model of human ovarian cancer cells in immunodeficient mice have used animals with ages ranging from 4 to 8 weeks [5, 9, 10]. In one experiment in which we injected 7-week old mice with AdXGH 4 and 8 days after tumor cell injection, we did not see an effect on survival (not shown), and we did not do further experiments with mice this age or younger.

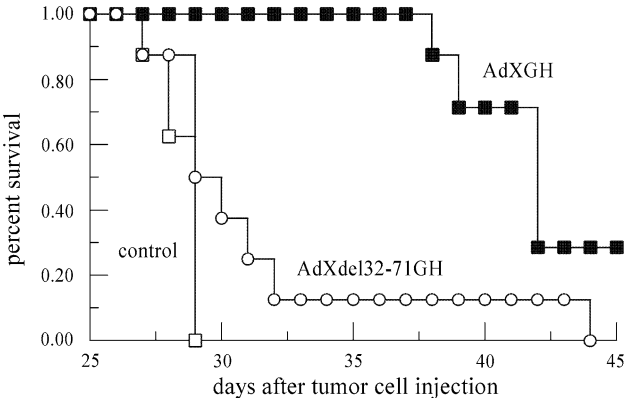

Fig. 1 Effect of ip administration of AdXGH or AdXdel32-71GH on survival of mice with SKOV3.ip1 tumors. SCID mice were injected with 5×10^6 SKOV3.ip1 cells each (day 0). Mice each received ip injections of PBS as controls (open squares, eight mice), 2×10^9 ifu AdXdel32-71GH (open circles, eight mice), or 2×10^9 ifu AdXGH (filled squares, seven mice) 4 and 8 days after tumor cell injections. One mouse in the group treated with AdXGH lived 141 days and one lived 350 days after tumor cell injection. Survival of AdXGH-treated mice was significantly different than that of controls ($P < 0.0003$)

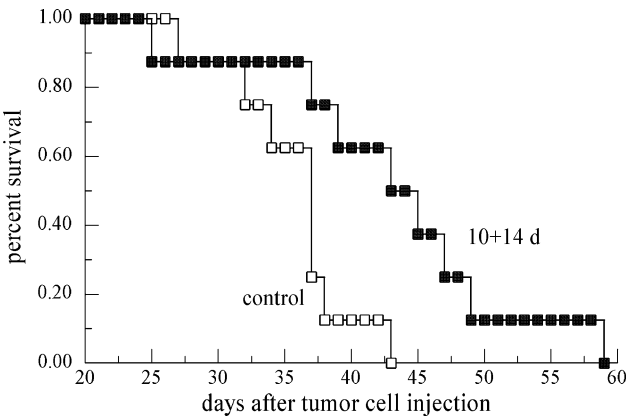

Fig. 2 Effect of ip administration of AdXGH given 10 and 14 days after SKOV3.ip1 tumor cell injection on survival of mice. SCID mice were injected with 5×10^6 SKOV3.ip1 cells each (day 0). Control mice (open squares, eight mice) and 2×10^9 ifu AdXGH 10 and 14 days after tumor cell injection (filled squares, eight mice). Survival of mice treated with AdXGH 10 and 14 days after tumor cell injection was significantly different than that of controls ($P < 0.016$)

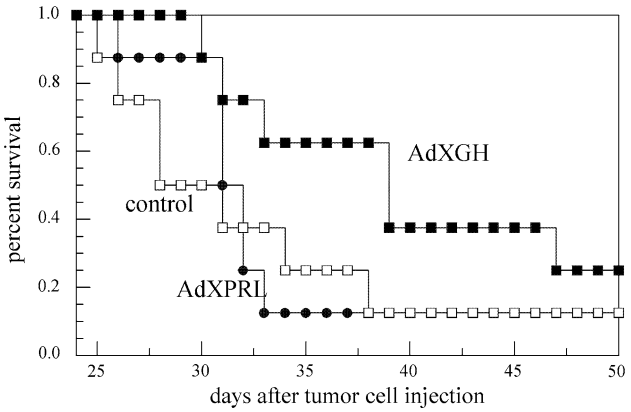

Fig. 3 Effect of ip administration of AdXGH or AdXPRL on survival of mice with SKOV3.ip1 tumors. SCID mice were injected with 5×10^6 SKOV3.ip1 cells (day 0). Control mice (open squares, eight mice), 2×10^9 ifu AdXGH 4 and 8 days after tumor cell injection (filled squares, eight mice), and 2×10^9 ifu AdXPRL 4 and 8 days after tumor cell injection (filled circles, eight mice). Mice that lived longer than 50 days: control 69 days, AdXPRL 123 days, and AdXGH 69 and 72 days

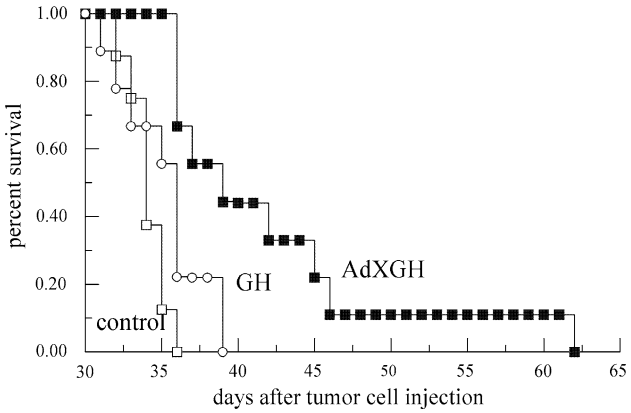

Fig. 4 Effect of multiple ip injections of human growth hormone or AdXGH on survival of mice with SKOV3.ip1 tumors. SCID mice were injected with 5×10^6 SKOV3.ip1 cells (day 0). Control (open squares, eight mice), 2×10^9 ifu AdXGH 4, 8, 12, and 16 days after tumor cell injection (filled squares, nine mice), mice injected with 0.04 mg human growth hormone 4, 6, 8, 10, 12, 14, 16, and 18 days after tumor cell injection (open circles, nine mice). The survival of mice injected with AdXGH was significantly different from control ($P < 0.0001$) and from those injected with growth hormone ($P < 0.014$)

In some of the experiments reported here, one or two mice lived for extended periods in control or treated groups, such as the two mice treated with AdXGH in Fig. 1. When there are long-lived mice in control groups, the difference in median survival between AdXGH treatment and controls is not statistically significant (for example, Fig. 3), although the increase in median survival always occurred; a lack of statistical significance for this reason occurred in three of the nine total experiments. There were more long-lived mice that had been treated with AdXGH than there were control mice. In the nine experiments, five of 49 mice treated with AdXGH at 4 and 8 days and one of 24 mice treated with AdXGH at 10 and 14 days lived for 100 days or more after tumor cell injection, but no control mice lived that long.

Lack of effectiveness of AdXPRL

Human growth hormone binds to both mouse growth hormone receptors and mouse prolactin receptors [11–13]. IGF I production, which is stimulated by growth hormone, has been implicated in stimulating human ovarian cancer [14, 15], so if the beneficial effects are mediated by binding to the prolactin receptor, using a virus vector containing prolactin sequences would be better. We compared treatment with AdXGH and AdXPRL. The median survival for control mice was 29 days, for AdXPRL-treated mice, 31 days, and for AdXGH mice, 39 days (Fig. 3). In this experiment, both control and AdXPRL-treated groups had one mouse that lived longer than 50 days, so the differences in median survival are not significant. AdXPRL treatment, however, did not cause an increase in median survival as large as the smallest increase in any experiment caused by AdXGH, which was 5 days (Fig. 4).

Serum levels of human growth hormone from mice treated with AdXGH 4 and 8 days after tumor cell injection were measured in samples collected 24 days after tumor cell injection, 20 days after the first AdXGH injection. The mean serum human growth hormone level was 60.5 ng/ml (range 17–174 ng, $n = 4$). The mean human growth hormone level in the peritoneal lavage was 1,680 ng (range 467–4,590 ng, $n = 4$). The ratios of the amount of human growth hormone in the peritoneal lavage to the concentration in the serum ranged from 7 to 150, so serum levels are only a rough reflection of the amount to which the ovarian tumors are exposed. In the experiment shown in Fig. 3, blood samples were taken from two mice in each group 24 days after tumor cell injection. Levels of human prolactin in mouse serum, 21 and 13 ng/ml, were lower than those of human growth hormone, 43 and 93 ng/ml, although the same amounts of virus were administered. No human growth hormone or human prolactin was detected in serum from control mice. A possible limitation of this experiment may be lower levels of human prolactin than human growth hormone in the mice.

Lack of effectiveness of human growth hormone administration without virus

Multiple injections of human growth hormone were unable to mimic the effects of AdXGH (Fig. 4). Mice that received human growth hormone every 2 days from 4 to 18 days after tumor cell injection had a median survival of 36 days,

not significantly different from controls, 34 days. Mice receiving AdXGH were given four injections of 2×10^9 ifu instead of the usual two. Their median survival was 39 days, significant compared to controls ($P < 0.0001$).

SCID mice do not destroy replication-deficient adenovirus because the mice are immunodeficient, so production of the protein encoded in the virus will remain detectable for weeks after infection [16, 17], and, as reported above, we found human growth hormone levels in the serum 20 days after the first AdXGH injection. Repeated injections of growth hormone do not give sustained growth hormone levels because the half-life of human growth hormone in mice is 4 min [18, 19], and so we developed pellets to supply sustained release of human growth hormone. Tablets prepared with 1.6 mg human growth hormone supply continuous levels for over 2 weeks when implanted in the peritoneal cavity of SCID mice injected with SKOV3.ip1 cells 4 days previously; the median serum level after 2 weeks of implantation was 19.7 ng/ml, with a range 4.2–29.2 ng/ml, $n = 4$ [20]. We implanted these tablets or tablets that contained no growth hormone in SCID mice 4 days after the injections with SKOV3.ip1 cells (Fig. 5). Control mice had a mean survival of 31.5 days, not significantly different from mice implanted with tablets containing no growth hormone, 34 days, or tablets containing growth hormone, 34 days. Mice that received two injections of AdXGH had a median survival of 41.5 days, significantly different from that of mice with growth hormone tablets ($P < 0.0112$) or that of control mice ($P < 0.0034$). We found similar results with tablets prepared with 0.8 mg human growth hormone; nine mice implanted with these tablets had a median survival of 31 days and eight control mice had a median survival of 30 days, not significantly different (survival curve not shown). One mouse implanted with these 0.8 mg tablets, however, lived more than 100 days, and no control mouse did. The median serum level in mice 15 days after they were implanted with these tablets was 13.2 ng/ml, with a range 8.3–19.5 ng/ml, $n = 3$ [20].

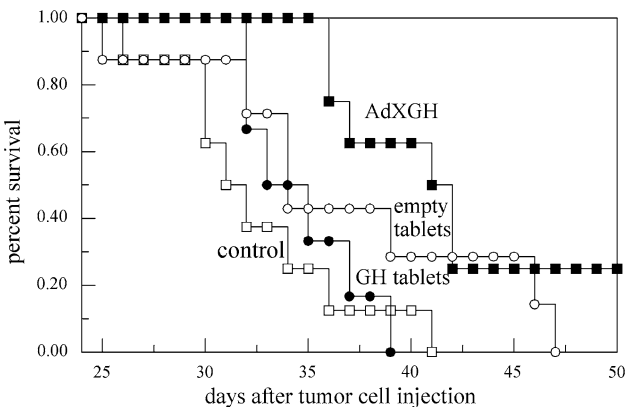

Fig. 5 Effect of ip implantation of a sustained release form of human growth hormone or AdXGH administration on survival of mice with SKOV3.ip1 tumors. SCID mice were injected with 5×10^6 SKOV3.ip1 cells (day 0). Control (open squares, eight mice), 2×10^9 ifu AdXGH 4 and 8 days after tumor cell injection (filled squares, eight mice), Growth hormone tablets implanted ip 4 days after tumor cell injection (filled circles, six mice), empty tablets implanted ip 4 days after tumor cell injection (open circles, seven mice). Median survival of mice treated with AdXGH is significantly different than that of mice with growth hormone tablets or control mice ($P < 0.0112$ and $P < 0.0034$, respectively). Two mice treated with AdXGH were euthanized 300 days after tumor cell injection

AdXGH treatment reduces tumor burden

Histopathology to assess tumor burden was performed on two sets of mice treated concurrently with two experiments in which survival was assessed. In each experiment, four control mice and four treated with AdXGH were euthanized for histopathology 24 days after tumor cell injection and are not part of the survival curves. In one experiment, mice were injected with AdXGH at 4 and 8 days after tumor cell injection in parallel with the experiment shown in Fig. 7. The control mice had macroscopic and microscopic evidence of numerous tumors throughout the peritoneal cavity that overlapped and could not be counted individually. There was occasionally invasion of tumor through serosa to the underlying tissues. All mice had liver metastases, and three of four mice had lung metastases. All four control mice were given a tumor burden score of 4. AdXGH-treated mice had fewer tumors in the peritoneal cavity and the tumors were discrete. One treated mouse had no macroscopic tumors. Two of the four treated mice were give a tumor burden score of 2 and two a score of 4. All four had minimal serosal/capsular fibrosis of abdominal organs. There were fewer invasions through the serosa, and none had lung metastases.

In the other experiment, one group was injected four times with AdXGH in parallel with the mice in the experiment shown in Fig. 4. The histopathology of the control mice in this experiment (Fig. 6a, c, e) was similar to the control group described above; two mice had lung metastases and all had liver metastases. These four control mice were also each given a tumor burden score of 4. Mice treated with AdXGH four times, however, had a greater reduction in tumor burden than with those treated twice, although survival was not longer than those treated twice. Two mice treated four times had macroscopic and microscopic tumors, one had only microscopic tumors and one had no evidence of tumors at all. The tumors, when detected, did not invade the serosa (Fig. 6b). The tumor burden scores were 0, 2, 2, and 3 for the four treated mice. None of the treated mice had lung or liver metastases.

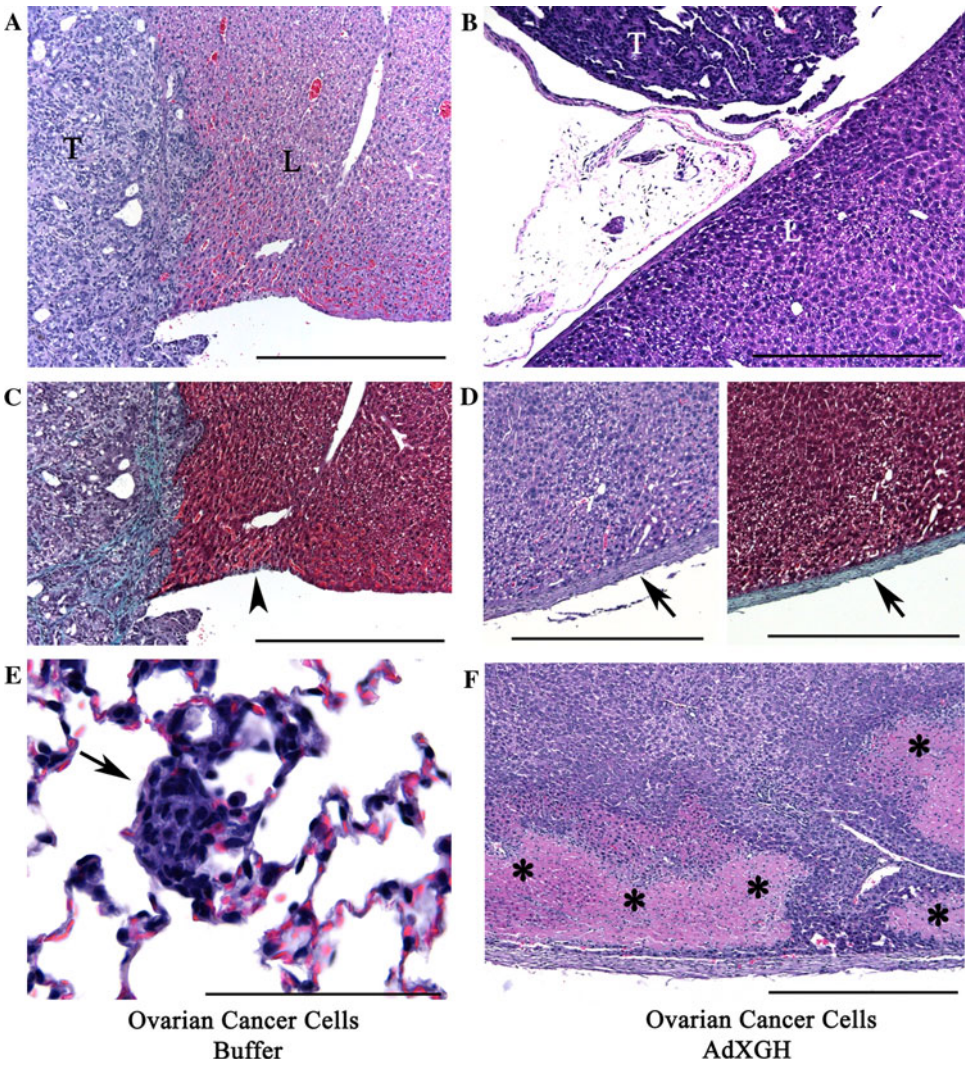

Fig. 6 Representative histopathology of mice 24 days after SKOV3.ip1 tumor cell injection, given buffer (**a**, **c**, **e**) or 4 ip injections of AdXGH (**b**, **d**, **f**). Livers from mice given buffer (**a**) had tumor (T) invasion deep into the liver (L) parenchyma with no capsular fibrosis (**c**, *arrowhead*), and had lung metastases (**e**, *arrow*). Livers from mice given four injections of AdXGH (**b**) had no tumor (T) invasion into the liver (L) parenchyma and no lung metastases (not shown), but did have capsular fibrosis (**d**, *arrows*). One mouse had multifocal hepatocellular necrosis (**f**, *asterisk*). Hematoxylin and eosin stain (**a**, **b**, **d** (*left panel*), **e**, **f**). Masson's Trichrome stain for collagen (blue) (**c**, **d** (*right panel*)). Scale bars: 500 μm (**a**–**d**, **f**); 200 μm (**e**)

All four mice treated four times with AdXGH had variable amounts of capsular and serosal fibrosis of all abdominal organs (Fig. 6d). All had hepatic enlargement with multifocal hepatocellular fatty changes that were absent in control mice, and one had marked multiple foci of hepatocellular coagulation necrosis (Fig. 6f). The mice that had been treated four times with AdXGH had more fibrosis and marked liver pathology.

Interaction of AdXGH with paclitaxel or carboplatin

Patients with ovarian cancer are usually treated with one of two drugs, paclitaxel or carboplatin [21]; AdXGH would be useful if it augmented current chemotherapy. We injected AdXGH 4 and 8 days and paclitaxel 5 and 12 days after SKOV3.ip1 tumor cell administration (Fig. 7). The median survival for controls was 37 days and treatments with either AdXGH or paclitaxel prolonged survival to a median of 46 and 85 days, respectively. The combination of AdXGH and paclitaxel, however, reduced the median survival to 29 days, so giving AdXGH before paclitaxel was not beneficial, since the combination was worse than paclitaxel alone. The treatment with AdXGH and then paclitaxel resulted in toxic effects in the intestinal track, since, after death, the small intestines in all mice treated with AdXGH and then paclitaxel were found to be packed with mouse chow.

We tested whether administering AdXGH after paclitaxel would prevent the unfavorable results that occurred when the virus was given before the drug. Paclitaxel was administered 5 days after SKOV3.ip1 tumor cell injection and AdXGH 10 and 14 days after tumor cell injection (Fig. 8). The median survival of controls was 27.5 days, of AdXGH-treated mice 33.5 days, and those treated with paclitaxel, 88 days. The combination of paclitaxel and AdXGH resulted in a median survival of 73.5 days, not significantly different from paclitaxel alone, so the combination of paclitaxel administered prior to AdXGH was

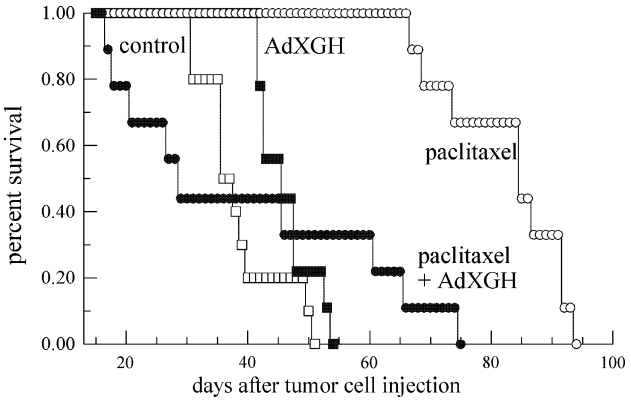

Fig. 7 Effect of ip administration of paclitaxel after AdXGH treatment on survival of mice with SKOV3.ip1 tumors. SCID mice were injected with 5×10^6 SKOV3.ip1 cells (day 0). Control mice (open squares, 10 mice), 2×10^9 ifu AdXGH 4 and 8 days after tumor cell injection (filled squares, nine mice), paclitaxel 5 and 12 days after tumor cell injection (open circles, nine mice), AdXGH 4 and 8 days after tumor cell injection and paclitaxel 5 and 12 days after tumor cell injection (closed circles, 9 mice). Survival of AdXGH-treated mice and paclitaxel-treated mice was significantly different from controls ($P < 0.028$ and $P < 0.001$, respectively), and survival of paclitaxel-treated mice was significantly different from survival of those treated with both ($P < 0.0001$)

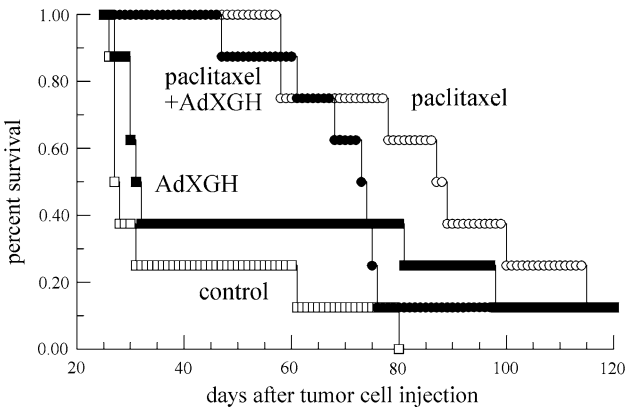

Fig. 8 Effect of ip administration of AdXGH after paclitaxel on survival of mice with SKOV3.ip1 tumors. SCID mice were injected with 5×10^6 SKOV3.ip1 cells (day 0). Control (open squares, eight mice), 2×10^9 ifu AdXGH 10 and 14 days after tumor cell injection (filled squares, eight mice), paclitaxel 5 days after tumor cell injection (open circles, eight mice), AdXGH 10 and 14 days after tumor cell injection and paclitaxel 5 days after tumor cell injection (filled circles, eight mice). Survival of paclitaxel-treated mice was significantly different from controls ($P < 0.0015$). One mouse each from the groups treated with AdXGH, paclitaxel, or both lived 449 days, at which point they were euthanized

not better than paclitaxel alone. The mice that were treated with paclitaxel and then AdXGH did not have excessive content in the small intestines when examined after death.

AdXGH treatment also did not improve the effects of carboplatin when given after this drug; mice received injections of carboplatin 5 days after tumor cell injection

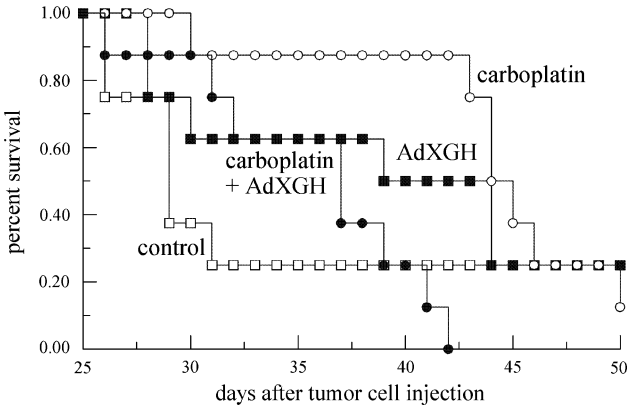

Fig. 9 Effect of ip administration of AdXGH after carboplatin on survival of mice with SKOV3.ip1 tumors. SCID mice were injected with 5×10^6 SKOV3.ip1 cells (day 0). Control (open squares, eight mice), 2×10^9 ifu AdXGH 10 and 14 days after tumor cell injection (filled squares, eight mice), carboplatin 5 days after tumor cell injection (open circles, 8 mice), and AdXGH 10 and 14 days and carboplatin 5 days after tumor cell injection (filled circles, eight mice). Survival of carboplatin-treated mice was significantly different than that of mice treated with both ($P < 0.0016$). Mice that lived longer than 50 days: control, 66 and 82 days; AdXGH 56 and 77 days; carboplatin, 103 days

and AdXGH 10 and 14 days after tumor cell injection. The median survival was 29 days and treatment with AdXGH or carboplatin prolonged survival to a median of 41.5 and 44.5 days, respectively. Administering AdXGH after carboplatin did not increase the effectiveness of carboplatin therapy; the mean survival of mice treated with both was 37 days (Fig. 9).

Discussion

We unexpectedly found that AdXGH administration prolongs median survival and reduces tumor burden in a mouse model of human ovarian cancer. The effect is specific for AdXGH, since AdXdel32-71GH and AdXPRL did not prolong survival. Others have also found a lack of effect of control adenovirus administration to SKOV3.ip1 tumors in the peritoneal cavity [22]. Administering growth hormone in a sustained release form, so that human growth hormone levels remain elevated for over 2 weeks did not increase the median survival. It is possible that it is the combination of virus infection and presence of human growth hormone that is beneficial.

The effect of AdXGH on survival was not large; survival was prolonged for an average of 9 days. The treatment would be potentially more important and easier to investigate if the effect were larger. Survival was not improved by doubling the dose of AdXGH, but there was a marked decrease in tumor metastases. The lack of improvement in survival is most likely caused by severe

liver damage, which occurs with the increased dose. Adenovirus is toxic to the liver if given intravenously and injection of virus into the peritoneal cavity, as we did, reduces the toxicity [23]. Increasing the number of injections of AdXGH apparently overcame the protection to the liver given by the intraperitoneal injections. Using an adenovirus less toxic to the liver [24, 25] may allow extended survival with the double dose of AdXGH that reflects the decrease in tumor burden.

Immunodeficient mice are used in this model of human ovarian cancer because normal mice will reject human cells based on species differences. SCID mice lack B and T cells when they are young, but some develop B and T cells as they mature, ranging from 2 to 10% of the number of B and T cells in normal mice [4, 26, 27]. Younger mice would be less likely to have B and T calls appear, but the one time we used mice that were 7 weeks old, we did not see a beneficial effect of AdXGH. SCID mice begin to mate between 8 and 10 weeks [28] and changes accompanying reproductive maturity may be necessary for the beneficial effects of AdXGH. One change that may have effects is production of estradiol, which regulates growth hormone actions [29].

In this model of human ovarian cancer, there were some long-lived survivors; a total of six out of 73 mice treated with AdXGH and one of 15 mice treated with human growth hormone tablets lived over 100 days, and no control mice did. Growth hormone injections have been reported to enhance immune activity [30, 31], so it may be that the long-lived survivors exist as a result of immune enhancement in leaky SCID mice.

Most women with ovarian cancer are given paclitaxel, carboplatin, or a combination of the two [21], so understanding how AdXGH treatment affects the efficacy of these agents is important. It was clear from visually observing the abdominal cavity of the mice that those given AdXGH and then paclitaxel had toxic effects on the intestinal track. Paclitaxel acts by arresting cells in mitosis and apoptosis follows; such effects are seen in the dividing cells of the intestine in addition to other tissue types [32, 33]. Growth hormone and IGF I, whose production is increased by growth hormone, increase division of cells in the intestine [34, 35], which will make the intestinal cells more susceptible to the toxic effects of paclitaxel. These interactions may explain why the mice treated first with AdXGH and then paclitaxel had intestinal blockage.

Carboplatin causes liver toxicity [36], as does adenovirus [23], which is a possible cause for the reduced effectiveness of the two combined. Expressing human growth hormone in a virus with less liver toxicity [24, 25, 37] may not only reduce tumor burden further and extend the survival period, but may allow AdXGH to be used after carboplatin treatment.

Little is published about the effects of growth hormone on ovarian cancer in women with the exception of investigations exploring whether an increase in IGF I, a growth factor whose production is stimulated by growth hormone, is associated with ovarian cancer [14, 15, 38, 39]. There is evidence for paracrine and autocrine actions of IGF I in ovarian cancer, but IGF I is usually regarded as a stimulator, rather than an inhibitor, of cell growth [38].

Necropsy results of mice injected with AdXGH four times showed that not only was tumor burden reduced, but that also there was a marked increase in fibrosis in the peritoneal cavity. The increase in fibrosis may be simply an adverse effect, but it is possible such a change may play a role in reducing tumor burden. Wang et al. [40] compared patterns of transcription in peritoneal tissue collected from patients with benign disease or ovarian cancer. In the samples from patients with cancer, they compared patterns from the peritoneum and attached stroma adjacent to tumors to samples not adjacent. Significant differences existed in expressed genes in the peritoneum from cancer patients and from those with benign disease [40]. In addition, samples from any one individual showed similar patterns regardless of location. These data indicate that the presence of ovarian cancer induces changes in gene expression that are similar throughout the peritoneum. Other evidence for changes in the peritoneum, which consists of a layer of mesothelial cells plus the underlying connective tissue, is that growth patterns of mesothelial cells in culture differ, depending on whether they come from women with advanced epithelial ovarian cancer or those with no cancer [41].

Cancer cells alter the cells and environment around them with the result that conditions are more favorable for growth and invasion [42–45]. An explanation for part of the beneficial actions of AdXGH may be that it modifies the peritoneum so that the cancer can no longer progress as it does in untreated mice.

Materials and methods

AdenoX-virus

Viruses containing the sequences for human wild-type growth hormone, human del32-71 growth hormone, or human prolactin were constructed using vectors for the Adeno-X Expression System (BD Biosciences). Primers 5′-cactctagacctgtggacagctcacac-3′ and 5′-tgtggtacctattaggacaaggctgctgct-3′ were used to add *Xba*I and *Kpn*I sites to human wild-type growth hormone and del32-71 growth hormone by polymerase chain reaction, and the sequences inserted into the multiple cloning site of the pShuttle vector. Human prolactin sequences were excised from

hPRLpcDNA3 [46], and inserted into pShuttle 2 using *Kpn*I and *Nhe*I restriction enzyme digestion. Sequences were removed from the Shuttle vector by digestion with PI-SceI and I-CeuI and ligated into Adeno-X viral DNA. Adeno-X DNA containing the inserts was transfected into HEK 293 cells after digestion with PacI using Superfect (Qiagen). High titer stocks were purified from early passage HEK 293 cells infected with lysates of secondary amplifications using Adeno-X virus purification kits (BD Biosciences). The purified virus was stored at −80°C in the formulation buffer supplied with the kits and a small portion frozen separately to use for titration. Virus titer was determined using Adeno-X Rapid titer kits (BD Biosciences). The particle number was 50–100 times the number of infectious particles in the preparations.

In culture, infection of SKOV3.ip1 cells with 10^6 ifu (infectious units) AdXGH caused the cells to produce 57 ± 15 ng human growth hormone in 24 h and infection with 10^6 ifu AdXPRL caused the cells to produce 17 ± 3 ng human prolactin in 24 h, each assayed by a chemiluminescent enzyme immunoassay (Immulite, Siemans). Del32-71GH is degraded intracellularly and nothing measurable is secreted [7]. Assays of serum samples of human prolactin and growth hormone were also performed with the Immulite assay. Blood was collected 24 days after tumor cell injection when animals were euthanized for histopathology, and lavage of the peritoneal cavity performed with 7 ml of PBS. The lower limit of detection was 0.05 ng/ml, and there was no detectable human growth hormone or prolactin in control mice, so cross reaction with mouse growth hormone was not evident.

SKOV3.ip1 cells

SKOV3.ip1 cells [3] were obtained from Drs. Ellen Vitetta and John Fulmer, UT Southwestern Medical Center, Dallas, TX, and cultured in alpha MEM plus 20% fetal bovine serum and 4 mM glutamine. Cells were prepared for injection into mice by removal from flasks by trypsinization and resuspension in medium, followed by centrifugation at 250*g* for 3 min and resuspension in sterile PBS at a concentration of 25 × 10^6 cells/ml.

Injection of mice

CB17 SCID female mice were from Charles River or Harlan Sprague-Dawley and maintained in specific pathogen-free housing in ventilated cages. Mice were fed a standard diet (Harlan TKD) in a 12/12 h light/dark cycle at 20–22°C. All injections were under sterile conditions. The Yale University Institutional Animal Care and Use Committee approved all protocols in compliance with the US Public Health Policy on Humane Care and Use of Laboratory Animals. All mice received 0.2 ml SKOV3.ip1 cells in PBS, 5 × 10^6 cells, on day 0 by injection into the peritoneal cavity. The formulation buffer in which the virus was frozen was replaced with PBS and the virus concentrated to 10^{10} ifu/ml using Centricon YM-50 Centrifugal Filter units (Millipore). Mice were injected ip with 0.2 ml virus solution or PBS 4 and 8 days after tumor cell injection unless otherwise indicated. Human growth hormone, lot AFP8996A, was obtained from the National Hormone and Peptide Program at NIH, dissolved in 0.01 M $NaHCO_3$ and diluted with PBS to give 0.25 mg/ml. Mice were each injected with 0.2 ml at the indicated times after tumor cell injection. Carboplatin (MP Biomedical), 0.2 ml of 2 mg/ml in PBS, or paclitaxel (MP Biomedical), 0.2 ml of 2 mg/ml in PBS, were injected ip at the indicated times.

Preparation of sustained release tablets

QA423L Biosynthetic 2-Cistron Human Growth Hormone Cysteine ex. dDAP, batch ID 274881 (date of manufacture 20/01/2007), with a HPSEC purity of 99.8% and water content of 4.8% w/w was provided by Lilly S.A. Poly(D,L-lactic-co-glycolic acid) (PLGA) (Resomer7 RG 504) with a molecular ratio of 50:50 was purchased from Boehringer Ingelheim, KG, Germany. All other reagents were those of analytical grade.

The formulation of PLGA tablets using 1.6 mg of freeze-dried recombinant human growth hormone (Lilly S.A.) and 80 mg of PLGA (Resomer7 RG 504) were prepared by direct compression in a Carver hydraulic press at room temperature with a force of 0.1 Tn during 30 s and a 6 mm test cylinder and pellet mold. The recombinant human growth hormone content in 10 tablets was determined by dissolving each one in tetrahydrofuran. Once the polymer was dissolved, the dispersion was centrifuged at 4,000 rpm for 10 min (Econospin Sorvall Instruments), the supernatant removed and residue vacuum-dried. This precipitate was dissolved in mobile phase and the hormone quantified measured by SEC-HPLC. The weight and thickness of each tablet was determined by a precision balance (Mettler Toledo, AG285) and a digital micrometer (Mitutoyo). The characterization of these tablets in vivo and in vitro is described in detail elsewhere [20].

Tablet implantation

All procedures were performed under sterile conditions. Mice were injected with carprofen, 5 mg/kg sc, before the operation. The anesthetic was 100 mg/kg ketamine and 10 mg/kg xylazine sc, followed by use of an isoflurane nose cone as necessary. Tablets containing growth hormone or empty tablets were implanted in the peritoneal cavity and the wall of the peritoneal cavity closed by

stitches. The skin layer was closed by staples, removed after 2 weeks. Carprofen, 5 mg/ml, was present in the drinking water for 3 days after the operation.

Survival curves

The median survival estimates were calculated using the Kaplan–Meier method, and log rank tests performed to determine significant differences using PRISM software (GraphPad Software, San Diego CA).

Histopathology

Mice (16 total, eight from each of two experiments) were necropsied, and all tissues harvested and examined with the observers blind to experimental manipulation; these mice were not part of the survival data. The abdominal cavity of each mouse was examined visually immediately after euthanasia by asphyxiation with carbon dioxide. The sternum and the head with the skull cap and skin removed were placed in Bouin's Fixative (VWR International, Batavia, IL) and the remaining tissues were placed in 10% Neutral Buffered Formalin (VWR International, Batavia, IL). A routine selection of tissues from all organ systems was processed, embedded in paraffin, sectioned at five microns, and stained with hematoxylin and eosin. All mice were examined visually at necropsy for macroscopic lesions and specifically for presence and spread of tumor throughout the abdominal cavity. All tissue sections were examined using light microscopy for pathologic changes and for microscopic evidence of tumor within the peritoneal cavity and metastases to the lung. Tumors were evaluated for invasion through the serosa or capsular surface into the abdominal viscera or diaphragm. All mice and tissues were evaluated grossly and by examination of stained slides at low and high power and scored for overall severity of tumor burden using a semiquantitative criterion-based methodology. Severity scores ranged from 0 to 4. Values of 0: none (within normal limits, tumors absent), 1: mild (1–3 masses within the abdominal cavity), 2: moderate (3–5 tumors), 3: marked ($\geq$5 tumors), 4: severe (tumor foci are too numerous to count, tumors invade or efface normal tissues, or tumors larger than 0.3 cm) were assigned according to presence and severity of tumor burden [47–49]. Digital light microscopic images were recorded using a Leica DM 5500B microscope (Bannockburn, IL), AxoCam MRC Camera and AxioVision 4.4 imaging software (Carl Zeiss Microimaging, Inc., Thornwood, USA) and optimized in Adobe Photoshop 8.0 (San Jose, CA).

Acknowledgments We thank Ms. Patricia Donovan and Julia Merk, who developed the surgical procedures for implanting the tablets; Patricia Donovan performed the implantations. The authors are grateful for rhGH provided by Lilly, with special thanks to Ms. Alison Kay, Mr. Vincente Hernández, and Ms. Carmen Bueno. The work in mice was supported by the Elsa U. Pardee Foundation, and we are grateful for their important support.

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