not examine sections of intestinal epithelium or bone marrow. Theoretically, any infection of nontumor cells by HSV 1 vectors may be attenuated by the administration of ganciclovir, which destroys HSV-infected cells.[6]

In summary, we demonstrated that the replication-competent HSV 1 vector hrR3 effectively destroys colon carcinoma cells *in vitro* and *in vivo*. The heterogeneity in susceptibility between cell lines and the absence of complete responses *in vivo* suggest the need for higher doses or more efficacious vectors. Several safety and toxicity issues need to be addressed before clinical trials.

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Discussion

DR. TIMOTHY J. EBERLEIN (Boston, Massachusetts): Metastatic disease from colorectal cancer, especially to the liver, is a major source of mortality. Chemotherapy is not effective, and therefore a new systemic type of therapy is required. This study attempts to address the problem by identifying a new treatment utilizing gene therapy. This study is a specific follow-up to Dr. Tanabe's earlier observations that colorectal carcinoma liver metastases have a much higher level of expression of ribonucleotide reductase compared to relatively nondividing human hepatocytes.

In these experiments the authors utilize a herpes simplex virus 1 vector deficient of ribonucleotide reductase. Since the vector lacks a functional ribonucleotide reductase gene, it preferentially will replicate in cells with high levels of endogenous ribonucleotide reductase. In this set of experiments Dr. Tanabe has shown reduction in tumor growth rate utilizing direct tumor injection. There appeared to be relatively little toxicity. However, this line of experiments raises several questions.

First, would a different route of injection, for example intravenous, reduce anti-tumor efficacy yet enhance toxicity, especially to gut epithelial cells and bone marrow cells, since they would very likely be affected by this vector?

Along the same line, most humans will possess antibodies to HSV 1 and therefore unless the vector is substantially changed will host immune response attenuate the efficacy of this vector in humans?

There appear to be a variability and susceptibility with this vector between different colon cancer cell lines. Do you have an explanation? Is this related to the particular cell line or the degree of differentiation of tumor, or perhaps the level of ribonucleotide reductase in the tumor?

In previous experiments as well as the experiments just provided, this vector therapy caused significantly reduced tumor growth and destruction of the tumor cells *in vitro*. Is this mechanism of destruction known? Specifically, how does this relate to the observation and the set of experiments that treatment with ganciclovir does not enhance reduction of tumor growth rate even in the control group? Is this a function of the tumor line chosen, the

HT 29, or is this a general phenomenon related to the mechanism of anti-tumor efficacy?

And finally, because there appears to be no long-term survival benefit and an absence of complete response, might this therapy be best utilized in the setting of combining it with a systemic chemotherapy or perhaps a regional therapy injected into the hepatic artery of the liver? Do you have preliminary data combining this treatment with any other modality?

DR. KENNETH K. TANABE (Boston, Massachusetts): To answer the first question regarding specificity of replication, the vector that we used for the study is deficient only in ribonucleotide reductase expression and therefore it replicates in tumors quite nicely, especially tumors in the liver and in the brain. This is because normal liver and normal brain tissue have minimal replicative activity compared to liver tumors and brain tumors. Nonetheless, as you point out there are other cells in the body that do have replicative activity, such as gut mucosa or bone marrow or hair follicles. These are essentially the same tissues that exhibit the most side effects from chemotherapy. We did not see hrR3 replication in those tissues.

The ability to detect herpes simplex virus is also a function of how hard you look for it. Looking for HSV simply by H and E staining and examining for cytopathic effects represents the least sensitive detection method. Histochemical staining for lacZ expression, as was done in this study is a little more sensitive. PCR amplification for herpes DNA is very sensitive, but may detect biologically inactive HSV. Primary cultures of bone marrow or gut mucosa on confluent monolayers of vero cells can be used to recover biologically active virus. In this study we only performed histochemical staining and did not detect any evidence of viral replication outside of the tumor after direct intra-tumoral hrR3 injection.

We have delivered this virus into the portal vein but didn't have time to show the data today. We observed excellent targeting to diffuse liver metastasis after regional delivery compared to intravenous delivery.

As you alluded to, unlike humans, mice don't have any preexisting antibodies to herpes. In addition, nude mice, such as the ones used for this particular study, have no cellular immunity. This underscores the fact that we are using a preclinical model. And I emphasize the word "model." Every model has its limitations. Most importantly, herpes simplex virus exhibits a very narrow natural host range. This virus causes disease only in humans and certain species of monkeys. Accordingly, all rodent models have serious drawbacks for the preclinical evaluation of herpes simplex viral vectors. We anticipate potentially different results in an immune-competent rodent model. In an immune competent rodent we may observe more anti-tumor activity, or we may observe less anti-tumor activity. However, I suggest that such results will be equally unhelpful and unable to predict results in humans. The toxicity of herpes simplex viral vectors can only be addressed in primates, and anti-tumor activity can only be addressed in humans.

With respect to the fact that some cell lines are very sensitive to the lytic effects of viral replication and others are less sensitive, I should point out these are relative differences. All of the cell lines that we have tested from humans are completely destroyed when as little as one viral pfu per one tumor cell are added. This is one to two log orders less than that required for adenovirus or vaccinia viruses. So although some cell lines are somewhat resistant, we are still talking about a very, very low multiplicity of infection.

Nonetheless, we are very interested in this area and are trying to figure out why or how tumor cells develop resistance. We have looked at p53 status and it does not influence resistance to herpes simplex virus.

DR. JOHN M. DALY (New York, New York): You have utilized tumor treatment using the herpes simplex type 1 because the virus replicates selectively in cells with high levels of endogenous ribonucleotide reductase, such as tumor cells. You have also demonstrated the presence of this enzyme in human colon carcinoma cell lines and its absence in human hepatocytes. You have demonstrated the viral cytolytic properties *in vitro*, and *in vivo* have demonstrated some decrease in the rate of tumor growth, with persistence of the virus for up to 40 days. It looks histologically as though it proceeds from the central aspects of the tumor out towards the periphery during that period of time, with disappearance at about 50 days. There are several questions I have about the model in this very good presentation.

Our lab and others have shown that when adenoviral vectors are injected into the murine flank tumor, systemic distribution actually occurs very rapidly. You have not demonstrated this. Is it simply because of the replication properties of the virus, or does it have to do with the timing of your investigation as to the presence of lacZ?

With such diffuse penetrance of the HSV type 1 throughout the tumor, why didn't ganciclovir work? Was that specific only to the HT29 tumor? Did you try any other tumor *in vivo* to test the true insertion of the thymidine kinase gene?

The next question is that the *in vivo* model tested only PBS against the replicating virus. Have you used nonreplicating HSV or another viral vector simply to test the hypothesis that the inflammatory response to the viral vector itself rather than gene insertion had something to do with the decrease in tumor growth rate?

Lastly, the hypothesis that you were interested in testing was to differentiate colon tumor from hepatocytes and then use either regional or systemic administration rather than tumoral administration. Can you tell us anything at all about the hepatic tumor implantation model, the use of systemic viral administration and its results in therapy?

DR. KENNETH K. TANABE (Boston, Massachusetts): We have not detected systemic herpes simplex virus. And as you point out, this may be related to when we look for it. We have looked for HSV at specific time points after introduction of the virus. We may miss it based on sampling error, especially if it is present only briefly in the systemic circulation. We hope that the specificity of HSV replication and the absence of systemic virus is because hrR3 is a replication-conditional virus that can only replicate in cells that express ribonucleotide reductase. The observed specificity may also be related to the fact that we are using a lower number of viral particles than are used in most *in vivo* adenoviral studies, where the inoculums may be as high as 10 to the 12th or 10 to the 13th particles of adenovirus.

There are several possible reasons why ganciclovir did not enhance the anti-tumor activity in this study. The first is that viral thymidine kinase mono-phosphorylates ganciclovir, but cellular enzymes are required to add a second and third phosphate. Ganciclovir tri-phosphate is the active metabolite. It may be that HT29 cells *in vivo* do not express the enzymes necessary to add the second and third phosphates. Second, unlike adenoviral gene therapy vehicles, this replicating herpes virus itself is cytolytic and

destroys infected cells very quickly. HSV gets in and within 16 to 18 hours it replicates and produces progeny virion and destroys the cell. This process may be so rapid that there is just not enough time to accumulate sufficient levels of viral thymidine kinase for ganciclovir to produce an additive effect. Third, these particular tumor cells may not form gap junctions. It has been demonstrated by other investigators that gap junctions are necessary for phosphorylated ganciclovir to be passed cell to cell in order to achieve some of the bystander effect.

In terms of using a different control virus, I think that is a wonderful suggestion. We do have some herpes viruses that are completely replication incompetent as opposed to replication conditional, and we could certainly use those to challenge or to test the hypothesis that you forwarded.

We have demonstrated in a model of diffuse liver metastasis that when hR3 is introduced into the portal vein it replicates specifically in the diffuse metastasis. When we compare portal vein delivery to intravenous delivery, we see much greater viral replication in the tumors after portal vein delivery and we also see more specific viral replication after portal vein delivery.

We would like to compare hepatic arterial delivery to portal vein delivery. But again one of the biggest problems is the species specificity of herpes simplex virus. Its natural host is limited to monkeys and humans. It is very difficult to find rodent cell lines that are susceptible to herpes viral replication. In contrast, all human cell lines are very susceptible. We essentially have been unable to find a rat carcinoma that is reasonably susceptible to lytic replication by herpes simplex virus in order to compare hepatic arterial delivery to portal venous delivery. We are now looking at survival studies, both in immuno-competent and immuno-incompetent animals using portal vein delivery of hrR3 to diffuse liver metastasis.