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Comparison of Two Different Methods for Inoculating VX2

Tumors in Rabbit Livers and Hind Limbs:

Clinical and Laboratory Investigations

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Abstract

Purpose—There are several competing methods to inoculate VX2 tumor in rabbit livers and hind limbs. The present report compared two methods to a) propagate VX2 cell strain in hind limbs, and b) inoculate liver parenchymal tumors in rabbits.

Materials and Methods—142 New Zealand white rabbits were used for this study (60 hind limb tumor (donor) rabbits; 82 liver tumor (recipient) rabbits). In the donor group (n=60), 9 rabbits received frozen VX2 cell suspension and 51 rabbits were injected with freshly prepared VX2 cell suspension. In the recipient group (n=82), 32 rabbits were injected with VX2 tumor cells while 50 rabbits were implanted with a small tumor fragment into the liver parenchyma. Success rates in terms of tumor growth were compared using Chi square or Fisher exact tests, with alpha=0.05.

Results—Hind limb and liver tumors were successfully grown in 48/60 (80%) and 57/82 (70%) rabbits respectively. Success rate of growing hind limb tumors increased from 33% (3/9) to 88% (45/51) when fresh VX2 cells instead of frozen were injected percutaneously (p<0.0011). Similarly the success rate for VX2 liver tumors almost doubled from 47% (15/32) to 84% (42/50) when a tumor fragment instead of VX2 cell suspension was used (p<0.00036). This also significantly reduced the incidence of metastasis (p<0.005).

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Conclusion—We recommend a) use of fresh VX2 cell suspension for percutaneous injection in the hind limbs of rabbits to maintain the VX2 cell strain, and b) surgical implantation of freshly harvested VX2 tumor fragment into the liver parenchyma to establish liver tumors.

Introduction

VX2 carcinoma is an anaplastic squamous cell carcinoma (SCC) derived from a virus-induced papilloma in rabbits. The VX2 tumor model, originally proposed by Shope and Hurst (1) in 1933, is used by interventional radiologists today for studies involving liver (2,3), kidney (4), head and neck (5), uterine (6) and lung (7) tumors. In the liver (8,9), the rabbit VX2 tumor model is widely used because a) its hepatic artery blood supply is similar to that of human liver tumors (10), b) it grows rapidly (9), c) it develops tumors of large enough size to be imaged (2), d) rabbits are large enough for effective catheter manipulation (2,11), and e) VX2, like advanced stage human tumors, is highly glycolytic, with elevated levels of mitochondrial bound hexokinase (12).

Despite its widespread experimental use, there remain several competing approaches to inoculate VX2 in the recipient rabbit liver and grow tumors within the donor rabbit hind limb. These methods include a) surgical implantation of small tumor pieces (13), b) open surgical injection of VX2 suspension cells (14,15), c) percutaneous injection via needles (16) or biopsy instrument (17); and d) infusion of VX2 carcinoma cells directly into hepatic artery or portal vein (18). These studies reported varying degrees of success in terms of tumor induction. Moreover, the question of using fresh vs. frozen (19,20) VX2 sample has never been addressed directly and remains unanswered.

In this study, using a sub-set of these methods, we aimed to determine the preferred method to a) propagate in-vivo VX2 cell lines in the hind limb of rabbits and b) inoculate VX2 into the rabbit liver parenchyma. For hind limb propagation, we compared injection of frozen versus fresh VX2 cell solution, while for liver tumor induction we compared surgical implantation of tumor fragments versus injection of VX2 cell solution. The significance of this study for future research is to improve the efficiency of tumor implantation, thereby potentially reducing the number of animals required for experiments, as well as animal expenses.

Material and Methods

Laboratory Setting and Animals

Our local institutional Animal Care and Use Committee approved all experiments. This 3-year retrospective study comprised of 142 New Zealand White rabbits, each weighing about 3.5–4.5 kg. Rabbits were divided into 2 categories: a) hind limb tumor (donor) rabbits (n=60), where the VX2 strain (NCI, Frederick, MD, USA) was maintained by serial percutaneous injection of the tumor homogenate into the thigh muscles of the rabbits, and b) liver tumor (recipient) rabbits (n=82), where VX2 cells were inoculated into the liver parenchyma. All surgeries and procedures were performed under proper anesthesia and sterile conditions.

Hind limb tumor (donor) rabbits and preparation of VX2 cell suspension

Hind limb tumor (donor) rabbits (n=60) were used to propagate and maintain the VX2 strain. Rabbits were anesthetized with intramuscular injection of 44-mg/kg ketamine hydrochloride and 3–5 mg/kg xylazine. Immediately before the procedure, lateral aspects of the hind limb of rabbits were locally shaved and disinfected using alcohol spray. With an 18-gauge needle, 0.75–1.0 mL of VX2 cell solution was injected deep in the gluteal muscles

of hind limbs of the rabbits. 9 rabbits received frozen VX2 cell suspension and 51 rabbits received freshly prepared VX2 cell suspension. 3 weeks after implantation, tumor growth was confirmed using magnetic resonance imaging (MRI). Animals were later sacrificed and hind limb tumors harvested.

All harvested tumors were processed immediately. VX2 tumor was cleaned from surrounding tissue, placed in a Petri dish, and kept on ice until harvest of cells. Tumor was then rinsed in the F-10 Nutrient Mixture (Ham) with L-glutamine (Invitrogen Corporation, CA). Tumor cells were scraped away from the surrounding tissue using a surgical blade and homogenized into a cell suspension. Grossly necrotic portions of tumor were removed. Cell suspension was filtered through 40- μ m mesh cell strainer (BD Biosciences, San Jose, CA) and pelleted at 500 g in a clinical centrifuge for 5 minutes. The supernatant was discarded and the cell pellet was re-suspended in equal volume of basic methylcellulose (MC) media (StemCell Technologies, BC, Canada). Samples were kept on ice until they were injected into the recipient rabbit's liver or hind limb or else frozen.

Implantation of VX2 tumors into liver parenchyma

All rabbits (n=82) in this group received liver VX2 tumor implantations. VX2 cell suspension was used in 32 rabbits while a 1mm³ tumor fragment from freshly harvested VX2 carcinoma was used to inoculate tumors in 50 rabbits. Out of the 32 rabbits which received VX2 cell suspension, 14 rabbits received frozen VX2 cell suspension and 18 rabbits received freshly prepared VX2 cell suspension.

For all surgical VX2 liver tumor implantations, each of the 82 rabbits were anesthetized with intramuscular (IM) ketamine 44 mg/kg, xylazine 3–5 mg/kg and administered inhaled isoflurane 2–3% as needed. Under aseptic conditions, a mini-laparotomy was performed in the subxiphoid area, exposing the liver. Using a 21-gauge needle, 0.2–0.4 mL of VX2 tumor cell suspension (frozen or fresh), was injected (Figure 1) slowly at 2 different locations, 4–5 cm apart and about 2 cm deep in the left anterior lobe of liver. Pressure was applied over the puncture site, using moist gauze for about 2 minutes. This helped us to achieve hemostasis and also prevented leakage of cancer cells.

In the other group, small tumor chunks (1mm³ fragment) (Figure 2a) freshly harvested from the donor rabbit were implanted in the liver of 50 recipient rabbits. Using a number-11 knife blade, 2 stab wounds were made 4–5 cm apart and about 2 cm deep in the liver parenchyma. Tumor pieces were then placed deep (Figure 2b) into both stab wounds. Gentle pressure was applied over the incision sites in liver to arrest bleeding and to prevent the fragment from getting dislodged. No attempt was made to suture the wounds; instead a small (1×1 cm²) piece of Surgicel (Ethicon, Johnson & Johnson, Somerville, NJ) was placed over the incision sites on liver (Figure 2c). We then closed the abdomen in 3 layers. Liver tumors were incubated for 3 weeks before imaging.

MRI

MRI was performed using a 1.5 T Magnetom Sonata clinical MRI scanner (Siemens Medical Solutions, Erlangen, Germany). Rabbits were imaged in the supine position using a flexible surface coil and remained intubated for isoflurane administration during imaging. Three weeks after tumor implantation, each rabbit underwent MRI to detect tumor growth. Tumor growth was considered positive when tumor was identified in axial and sagittal imaging planes by two independent MR imaging specialists.

Anatomic images of the hind limb or liver tumors in all 142 rabbits were acquired using a T2-weighted turbo spin-echo (T2W TSE) sequence with the following imaging parameters: TR/TE = 3000/82 milliseconds, 4-mm slice thickness, 130-Hz/pixel BW, 200 × 100 mm²

field of view, 256×126 matrix ($0.8 \times 0.8 \times 4.0$ -mm³ voxel size), turbo factor = 7, averages = 4.

Necropsy and Histopathology

Each rabbit was sacrificed with an intravenous injection of 150–200 mg/kg sodium pentobarbital (Euthasol; Delmarva Laboratories, Midlothian, VA) once MRI was completed. All liver tumors were harvested for pathologic confirmation of VX2 tumor growth with hematoxylin and eosin stain. Histological analysis of hind limb tumors was not performed because these tumors' only purpose was to maintain the VX2 cell line. Any rabbit that died prematurely underwent a full necropsy to determine the cause of death.

Data analysis

Competing methods of tumor inoculation were compared for success rate of tumor growth and incidence of death due to metastasis. Success rate was defined as presence of tumor ≥ 1 cm in diameter on MR imaging. The 1-cm size cut-off value was selected because this diameter was considered large enough to be confidently detected on MR imaging and to be useful for interventional procedures.

For each tumor inoculation method, we separately calculated success and death rates. 95% confidence intervals (CI) were also calculated for the difference in the success and death rates for different compared groups. Chi squared test was used to determine significant statistical differences in the compared groups. However, when the sample size was too small to use the Chi squared test, we used the Fisher exact test instead. Statistical significance was set at the $p < 0.05$ level.

Results

All 142 rabbits (donor and recipient) successfully tolerated VX2 tumor implantations. No adverse incident or death was observed at the time of inoculation. Tumor growth was successfully confirmed on MR imaging (Figure 3) after 3 weeks of incubation in 105/142 rabbits (74%). Typical size of hind limb tumors ranged from 4.0–5.0 cm while liver tumors were 1.5–2.0 cm in size. All tumors were confirmed at necropsy (Figure 4) and all liver tumors were further confirmed by histopathological examination (Figure 5) with hematoxylin and eosin stain.

Hind limb tumor (donor) rabbits

Hind limb tumors were grown in 48/60 rabbits, the overall success rate in this group being 80%. Tumors were seen in 3/9 rabbits (33%) when frozen VX2 cells were used and in 45/51 rabbits (88%) when fresh VX2 cells were injected. The difference in success rates between the two methods was 55% (95% CI, 17% to 80%). Results were statistically significant with $p < 0.0011$ (Fisher exact test). No rabbits died in this group. Results from the hind limb tumor (donor) rabbits are summarized in Table 1.

Liver tumor (recipient) rabbits

Liver tumors were grown in 57/82 rabbits with an overall success rate of 70%. Tumors were grown in 15/32 rabbits (47%) when VX2 cell suspension was used. On further sub-analysis, tumors were seen in 5/14 rabbits (36%) when frozen VX2 cells were used and in 10/18 rabbits (56%) when fresh VX2 cells were injected.

We successfully grew tumors in 42/50 rabbits (84%) when VX2 tumor fragment was used for liver tumor implantation, rather than the cell suspension.

The difference in success rates between the two methods (injecting VX2 cell suspension vs. tumor fragment inoculation) was 37% (95% CI, 14% to 56%). Results were statistically significant (Chi square=12.69, $p=0.00036$).

7/32 rabbits (22%) died when VX2 cell suspension was used, while only 1/50 rabbits (2.0%) died when tumor fragment was implanted. All the deaths occurred during MR imaging. The difference in the death rate was 20% (95% CI, 4.0% to 39%), which was statistically significant at $p<0.005$ (Fisher exact test). We attribute all deaths to compromised pulmonary function/reserve from lung metastasis (Figure 6), as confirmed by necropsy. The clinical significance of this reduced lung function was amplified by general endotracheal anesthesia and accounted for the deaths during MR imaging. Results from the liver tumor (recipient) rabbits are summarized in Table 2.

Discussion

In this animal study, we successfully demonstrated the superiority of using fresh over frozen VX2 material for propagation of the donor hind limb model, as well as for induction of liver tumors in recipient rabbits. We also showed that implanting a chunk of VX2 tumor in the rabbit liver significantly improves the success rate of tumor growth over VX2 cell solution, while reducing the incidence of lung metastases.

Implanting the tumor percutaneously in the hind limbs of the rabbit is the most common and widely accepted method of maintaining the VX2 strain. Although Thorstensen et al (17) grew VX2 strain intraperitoneally in rabbits, they did not mention their success rate of tumor growth. In our study, the success rate of propagating in vivo VX2 tumor in the hind limbs of donor rabbits increased significantly to 88 % when we used fresh VX2 cell suspension as compared to 33% when frozen VX2 cell suspension was used. Oya et al (19) and Shomura et al (20) used frozen samples for implanting liver and intra-pulmonary VX2 tumors respectively but neither of them mention their success rate nor did we come across any other reference which directly addressed the question of using fresh vs. frozen samples.

The success rate of implanting liver parenchymal VX2 tumors almost doubled from 47% to 84% when we used fresh tumor fragments instead of VX2 cell suspension. Use of VX2 cell suspension resulted in a higher failure rate for tumor implantation, and a statistically increased incidence of lung metastasis. Failure rate was even higher when frozen VX2 cells were injected. We attribute these poor results with frozen VX2 suspension to reduced viral titers in the frozen aliquots over a period of time. The increased incidence of metastasis in the same group is most likely related to inadvertent intravascular injection during the intended liver parenchyma implantation. This inadvertent intravascular injection occurred, despite our technique of aspirating the needle prior to injection to ensure no blood return.

In a previous study that tested methods to induce VX2 liver tumors, Chen et al (13) compared injection of VX2 solution vs. surgical placement of VX2 tumor fragments. They achieved good success rate in terms of tumor growth by implanting VX2 tumor fragments in the liver, however they implanted the tumors only superficially in the sub capsule of the left lobe of liver. Our surgical technique of implanting VX2 tumors deep in the liver parenchyma using a scalpel completely differs from their superficial sub-capsular implantation method using a band. Our parenchymal location also more closely mimics the location of most clinical liver tumors. Finally, compared to the study by Chen et al (13), we used a larger sample size of rabbits with liver tumors (82 vs. 40 rabbits) and additionally compared fresh vs. frozen samples.

We think that surgical implantation of a fresh tumor fragment directly in the liver (13) is preferable to injection of VX2 suspension cells or infusion of VX2 carcinoma cells directly

into hepatic artery or portal vein. Surgical implantation of tumor fragment more effectively controls the site of tumor growth, while tending to induce tumors that are more spherical in shape with well-delineated margins as compared to the method of injecting VX2 cell suspension. The incidence of lung metastasis and death are also considerably reduced. In our study, only one rabbit that underwent surgical VX2 tumor implantation died prematurely from necropsy-proven lung metastasis.

However, the surgical method of implanting tumor fragments in liver also has its own disadvantages. First, it is more traumatic and risks surgical complications like infection, hemorrhage, or bile leak. These risks can be minimized by taking proper pre-, intra- and post-operative precautions. Second, the tumor may not grow when necrotic tissue, instead of viable tumor cells are implanted into the liver. This issue can be avoided by selecting the fragment from the periphery of the donor tumor where most of the viable tumor cells are likely present. Finally, the implanted tumor fragment in the liver can fall out in the peritoneal cavity and may start growing at a different site. The chances of this happening can be reduced by applying manual pressure and a piece of Surgicel or similar absorbable hemostat over the liver incision site.

There were several important limitations to our study. First, experimental constraints and variable success rates led to an unequal number of rabbits in all the groups. Second, we did not compare all published methods of VX2 tumor implantation. However, we choose common previously described approaches. Finally, the results from our study may not be generalizable to other organs, such as the uterus (6), larynx (21), brain (5) or lung (20).

In conclusion, we recommend a) use of fresh VX2 cell suspension for percutaneous injection in the hind limbs of rabbits to maintain the VX2 cell strain, and b) surgical implantation of freshly harvested VX2 tumor fragment into the liver parenchyma to generate liver tumors. By applying these approaches, interventional radiologists can potentially enhance their success rates for using this helpful tumor model.

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Figure 1.
VX2 tumor cell suspension injection. Photograph shows the injection of VX2 tumor cell suspension into the liver parenchyma.

Figure 2. VX2 tumor fragment implantation. (a) Photograph shows a 1mm³ VX2 tumor fragment (arrow) from a donor rabbit being implanted deep (b) into the liver parenchyma. (c) A 1×1 cm piece of Surgicel (absorbable hemostat) placed over the incision site (arrow) on liver.

Figure 3.

(a) T2-weighted MR image of the hind limbs of rabbit showing tumors (arrows) in both the hind limbs. (b) T2-weighted MR image showing 2 discrete tumors (arrows) in the left lobe of the rabbit's liver.

Figure 4. Gross images of the liver and hind limb tumors. Photographs show (a) Multiple tumors of varying sizes spread all over the left lobe of the liver, 3 weeks after injection of VX2 solution. (b) Two discrete masses (arrows) about 2×2 cm in size in the liver, 3 weeks after implantation of a fragment of VX2 carcinoma. (c) VX2 tumor specimens dissected from the hind limb of donor rabbit. Note the necrotic region (arrow) in the center of the tumor.

Figure 5. Histopathologic image (hematoxylin-eosin stain; 2.5x magnification), demonstrates the well-defined interface (arrows) between the inner VX2 tumor and the surrounding normal liver parenchyma in the rabbit liver implanted with a VX2 tumor fragment.

Figure 6.
Gross image of rabbit lungs shows multiple bilateral metastases. This rabbit was inoculated in the liver with VX2 cell suspension.

Table 1

Result of inoculating VX2 carcinoma cells into the hind limb of donor rabbits (n=60)

	N	Tumors (Success rate)	No Tumors	Death
Injecting Frozen VX2 cells	9	3 (33.3%)	6	0
Injecting Fresh VX2 cells	51	45 (88.2%)	6	0
Total	60	48 (80%)	12	0

(P=0.0011, Fischer exact test)

Table 2

Result of implanting VX2 carcinoma cells or tumor fragment in the liver of recipient rabbits (n=82)

	N	Tumors (Success rate)	No Tumors	Death
Injecting VX2 cells	32	15 (46.8%)	17	7 (21.8%)
<i>Subcategory:</i>				
<i>Frozen:</i>	14	5 (35.5%)	9	3 (21.4%)
<i>Fresh:</i>	18	10 (55.5%)	8	4 (22.2%)
Implanting VX2 tumor fragment	50	42 (84%)	8	1 (2%)
Total	82	57 (69.5%)	25	8 (9.7%)

(Chi-square=12.69, p=0.00036).