Perivascular and intralesional tissue necrosis after hepatic cryoablation: Results in a porcine model


Background. Cryosurgical ablation of malignant hepatic tumors is being increasingly used for definitive treatment of metastatic colorectal and primary hepatic tumors. The lack of tumor necrosis near vessels that result from inadequate freezing may contribute to local recurrence and thus limit the applications of this therapy. This study was designed to determine whether single-freeze cryoablation could cause necrosis of both the perivascular and intralesional hepatic parenchyma.

Methods. Ten pigs were treated with one 15-minute cycle of cryoablation. Five additional animals were treated with overlapping cryolesions to simulate a double freeze. After 24 hours, animals underwent reoperation with portal vein cannulation and infusion of formalin. Serial sectioning and hematoxylin and eosin staining of cryolesions were performed.

Results. Complete cell death was visualized within all cryolesions. There was no difference between once- or twice-frozen tissue. Vessels within or adjacent to cryolesions showed necrosis of hepatic tissue up to the vessel wall. No sections revealed incomplete necrosis of perivascular hepatic parenchyma.

Conclusions. Single-freeze cryoablation results in necrosis of intralesional hepatic parenchyma without added benefit from repeat freezing. Complete necrosis of the perivascular tissue suggests that cryosurgical ablation can effectively cause necrosis immediately adjacent to vessels without concerns of incomplete ablation resulting from the heat sink effect. (Surgery 1997:122:742-7.)

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Surgical resection of primary and metastatic liver tumors is possible in only 5% to 10% of cases.¹ Options for hepatic resection are limited when lesions involve multiple lobes, are near major vascular structures, or are present in patients with limited hepatic reserve or recurrence after a prior resection. To increase the number of patients amenable to surgical therapy, cryosurgical ablation is being used increasingly either as definitive therapy or as an adjunct to surgical resection.² However, clinical use of hepatic cryosurgery preceded extensive testing in experimental animal models. Because of the lack of experimental data, differences in the approach to cryoablation have developed, including use of repeat freeze/thaw cycles. Clinically, a freeze/thaw/freeze cycle is most often used.³,⁵

Local recurrence after technically successful cryosurgical tumor ablation, the most significant problem limiting the use of this technology, may be due to the lack of necrosis near vessels secondary to the continual flow of warm blood. This has been referred to as the heat sink effect.⁵ Although this effect has been proposed as a significant limitation to hepatic cryosurgery, it has not been studied in an animal model. We hypothesized that single-freeze cryosurgical ablation could result in necrosis of hepatic parenchyma adjacent to vessel walls as well as within the cryolesion. Objectives of this study were (1) to assess the degree of histologic tissue necrosis in the perivascular hepatic parenchyma after single-freeze cryoablation and (2) to compare the histologic difference between hepatic tissue receiving a single freeze versus tissue subjected to a freeze/thaw/freeze cycle.

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Fig. 1. A crisp demarcation between normal hepatic parenchyma (upper left portion of this section) and necrotic tissue (lower left) was visualized with a thin band of neutrophils at the border. Note the preserved vessel wall on the right of this section (original magnification ×200).

Fig. 2. Gross appearance of sectioned liver from overlapping cryolesions, simulating a double freeze in the area of overlap. Fusion of the necrotic area from tissue receiving one and two freezes is seen centrally. There is no gross difference between these areas.

Table. Observations on vessels examined histologically

<table>
<thead>
<tr>
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<th>Peripheral portion of cryolesion</th>
<th>Central portion of cryolesion</th>
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<tbody>
<tr>
<td>Number of vessels examined</td>
<td>41</td>
<td>98</td>
</tr>
<tr>
<td>Number of vessels thrombosed</td>
<td>9 (22%)</td>
<td>49 (50%)</td>
</tr>
<tr>
<td>Necrosis of perivascular hepatic parenchyma visualized</td>
<td>41 (100%)</td>
<td>98 (100%)</td>
</tr>
</tbody>
</table>

The peripheral portion of the cryolesion refers to an area 2.3 mm from the border of necrosis.

MATERIAL AND METHODS

Animals and surgery. Fifteen normal swine (mean weight, 28.3 kg) were used for this study. Approval for this protocol was obtained from the University of Wisconsin research animal use committee. General anesthesia was induced with an intramuscular injection of telazolamine and zolazepam (Telazol; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (Rompun; Bayer Corporation, Shawnee Mission, KS) and maintained with inhaled halothane. Animals were prepped with a 10% povidone-iodine solution and a bilateral subcostal incision was performed for liver exposure.

Single freeze cryolesions. In 10 animals, 3.4 mm cryoprobes were placed into the hepatic parenchyma according to the Seldinger technique (Onik-Cohen percutaneous access kit, Cook Urological, Spencer, IN). One 15-minute cycle of cryoablation at 100% freezing capacity was performed under ultrasound guidance using an Aloka SSD-2000 7.5 MHz linear intraoperative transducer (Aloka Ultrasound, Wallingford, CT). Two lobes were treated in each animal, resulting in 20 cryolesions. Six of these lesions were made with a standard nitrogen-based system (Accuprobe 450SP, Cryomedical Sciences, Inc., Rockville, MD). The remaining 14 cryolesions were created using an argon-based system that functions using the Joule-Thompson principle (Cryocare System, Endocare, Inc., Irvine, CA).

Double freeze cryolesions. In five animals, a single cryolesion was created with a 15-minute freeze. The cryolesion was passively thawed for 10 to 15 minutes until the frozen tissue had receded grossly from the edge of the cryolesion. After thawing, a second probe was placed adjacent to the first and another cryolesion performed, resulting in an area of overlap of the two cryolesions. This method simulated a double freeze in the overlapping area of the cryolesions, with tissue receiving a single freeze immediately adjacent to that receiving two freeze cycles. This model duplicated the two cycles of cryoablation that are performed clinically.

Tissue harvest and histopathologic study. After 24 hours, animals were anesthetized as described previously and underwent reoperation. The portal vein was cannulated and an intravenous bolus of heparin (2,000 units) administered. The animal was then sacrificed by intravenous infusion of pentobarbital and phenytoin (Beuthanasia-D, Schering-
the cryolesion and surrounding normal hepatic parenchyma. The liver was then removed en bloc.

The liver was sectioned at 4 mm intervals along the transverse axis of the cryoprobe. Representative tissue samples were mounted in paraffin blocks, sectioned at 10 μm and stained with hematoxylin and cosin. Gross examination and tissue sections were performed at the central region and borders of the visible cryolesion. In the five animals receiving a double freeze, sections were obtained from those areas that were subjected to both a single and a double freeze.

Sections of 41 vessels from just inside the border of the cryolesion (within 2.5 mm) and 98 vessels from the central portion of the cryolesion were examined. Vessels were identified as either patent or thrombosed. If fibrinous material with disintegrating red blood cells was present within the lumen, the vessel was deemed thrombosed. However, if the lumen was either empty or filled with discrete round red blood cells, the vessel was considered patent.

After sectioning, normal hepatic parenchyma was removed from around the cryolesion, and the necrotic volume was weighed. The reproducibility of cryolesions both within and between animals was compared via measurement of the weight and diameter of the necrotic tissue, as well as by its gross appearance.

RESULTS

All animals survived initial treatment without hemorrhage or cracking of the liver surface. One animal remained ventilator dependent after surgery and was reexplored. No hemorrhage or intraabdominal etiology was identified. This animal was sacrificed 6 hours after cryoablation. All other animals were sacrificed at 24 hours.

Mean weights of single-freeze cryolesions for the nitrogen-based and argon-based devices were 21.88 ± 10.75 gm and 23.9 ± 7.86 gm, respectively, (p = 0.121, paired t test). Mean diameters were 2.63 ± 0.89 cm and 2.9 ± 0.87 cm, respectively (p = 0.009, paired t test). There was no gross or histologic difference between cryolesions produced by the two devices.

At gross examination, the borders of cryolesions appeared white with a central hemorrhagic necrotic area. Histologic evaluation demonstrated coagulative necrosis of hepatic parenchyma with a crisp demarcation between normal and necrotic tissue (Fig. 1). No sections revealed incomplete necrosis within the cryolesion after a single freeze. A thin band of neutrophils approximately 2.0 mm wide was present at the border of the cryolesion. No gross or histologic damage was seen outside the

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**Fig. 3.** Whole mount of tissue that received two cycles of cryoablation reveals no histologic difference between tissue receiving one or two freeze cycles (*). Arrows point to the border between necrotic tissue and normal hepatic parenchyma. Complete homogenous necrosis is visualized throughout the area that received either one or two freeze cycles with no sections revealing incomplete necrosis. These findings were verified under high power microscopy.

**Fig. 4.** Patent vessels with perivascular necrosis up to the vessel (original magnification ×200).

Plough, Kenilworth, NJ). Immediately following sacrifice, the portal vein was infused with 10% neutral buffered formalin to maximize preservation of
cryolesion. In areas receiving two freeze cycles, there were no gross (Fig. 2) or histologic (Fig. 3) differences between tissue receiving one or two freeze cycles.

All sections of vessel walls showed necrosis of hepatic tissue up to the adventitia, with relative sparing of the vessel wall (Figs. 4 and 5). Vessel diameters ranged from 0.1 to 4.5 mm after preservation. Thrombosis of vessels occurred in nine of 41 (22%) vessels from the periphery of the cryolesion and 49 of 98 (50%) vessels located in the central portion of the cryolesion ($p = 0.002$, chi-squared) (Table). No sections revealed incomplete necrosis of hepatic parenchyma adjacent to vessel walls.

Gross examination of cryolesions demonstrated deflection of the iceball away from large blood vessels in six cases. Vessels that caused this deflection ranged from 2 to 6 mm in diameter (mean 3.77 ± 1.36 mm). In the other 14 lesions, the gross shape of the iceball was reproducible between systems and animals. As a means for comparison, 15 vessels of similar size (mean 3.89 ± 0.81 mm, range 3 to 5.5 mm) were examined in cryolesions that did not have deflection of the iceball. All vessels that resulted in deflection of the cryolesion were patent. However, in the 15 vessels we examined from normal-appearing iceballs, only 6 (40%) were patent ($p = 0.012$, chi-squared).

**DISCUSSION**

Proposed mechanisms of tumor destruction by freezing include intracellular ice crystal formation, osmotic changes during thawing, and local tissue ischemia as a result of vascular thrombosis. Studies of isolated cell suspensions have identified some of the factors responsible for cell death after freezing, and it is now clear that multiple freezes, lower temperatures, and increased rates of freezing all contribute to a smaller proportion of surviving cells post-freeze. Less clear is the relationship of these factors when applied to solid organs in vivo. Freezing of cell suspensions underestimates the degree of necrosis in frozen tissue largely because of the lack of tissue ischemia.

One limitation of this study is that single freeze cryoablation was tested in normal pig liver, rather than using a tumor model. Other studies have found that histologic changes after freezing are similar in normal animals and in small animal tumor models, with complete necrosis of tumor after single freeze cryoablation. However, the histologic appearance of the perivascular tissue after a single freeze has not been examined in a tumor model. Further studies to verify the histology of the perivascular tissue in a tumor model after cryoablation are necessary.

Published data regarding histologic appearance and kill rates after single versus multiple freeze cycles are controversial. In one study, cryoablation of rat colon cancer isografts to the thigh resulted in 100% kill after multiple freeze cycles, but viable cells were present in one lesion that received a single 8-minute freeze. Although this model used a subcutaneously implanted tumor, the authors concluded that a single freeze was inadequate in hepatic cryosurgery of colorectal metastases. Unfortunately, the cryolesion was not monitored by ultrasound, and, more important, it is unclear whether the freeze zone completely encompassed the tumor. Multiple freeze cycles have been shown to increase the volume of frozen tissue, decrease the rate of thawing, and to increase the radius of necrotic tissue when compared to a single freeze. More significantly, all cryolesions in one study showed complete cell death after a single freeze.

The hepatic necrosis we saw after 24 hours using a single freeze is supported by a previous study in which complete tissue ablation was found on postoperative day 2 after a single freeze. There were no areas of nonhomogeneous necrosis within our specimens. We tested the hypothesis that single-freeze cryoablation could result in cell death by overlapping repeat freezes to simulate a freeze/thaw/freeze cycle. Overlapping the two freeze zones rather than freezing twice with the same cryoprobe created a rigorous test for comparing once-frozen to twice-frozen tissue, partially because the
periphery of the cryolesion is the warmest area during freezing and, therefore, the most likely area for incomplete tissue death to be expected. Also, if we had performed two freezes with one cryoprobe, it would have been difficult to determine which part of the tissue received one freeze and which part received two. Using this model, no gross or histologic difference was observed when comparing tissue that received one freeze to tissue that received two freezes. Data that shows improved kill rates with multiple freezes may be due to lack of adequate margins with a single freeze. The second freeze cycle increases the diameter and volume of ablated tissue and should thus yield a more substantial surgical margin. The concept of using two freeze cycles to enhance tissue destruction within the cryolesion is not supported by this experiment.

There is little experimental data regarding perivascular tissue necrosis in humans or animal models, although concerns of local recurrence resulting from incomplete ablation near vessels continues.16 When blood flow was occluded, vessel walls were found to be resistant to structural change after freezing in a dog, with no episodes of rupture and little evidence of damage on histologic examination.17 However, this study did not examine perivascular tissue but rather freezing to isolated vessels only. It is likely that vessel walls are more resistant to freezing because of the increased amounts of fibrous tissue present. More important than examination of the vessel wall, we found histologic evidence of complete homogenous necrosis of the perivascular hepatic parenchyma after single-freeze cryoablation on all sections, regardless of vessel size.

Deflection of the gross shape of the iceball when freezing near large vessels has not previously been described. One explanation may be that as the expanding iceball comes into contact with a major vessel, the internal thermosensor in the probe tip registers increased temperatures. The flow of liquid nitrogen or argon gas is continued to achieve the desired temperature. The cryolesion cannot expand where it is in contact with a vessel with active blood flow and, therefore, grows disproportionately on the contralateral side, creating an iceball that appears deflected. This finding was not appreciated by ultrasound in our study. Because ultrasound is a real-time modality without a fixed plane of reference, the examiner is able to unknowingly move the probe to make the iceball appear symmetric. The danger is that large amounts of tissue on the contralateral edge of the iceball may be inadvertently frozen. This may be important when attempting to freeze a tumor that has encompassed a vessel along a substantial portion of its circumference unless multiple probes, placed in close proximity, are used.

In summary, this study has demonstrated the ability of current cryosurgical equipment to cause complete, homogenous tissue necrosis using a single freeze in a porcine model. No difference was detected upon histologic examination when a second freeze was applied to the same tissue. Therefore, we question the clinical doctrine of using a freeze/thaw/freeze cycle if an adequate margin can be accomplished. We have also demonstrated the ability of cryosurgery to cause necrosis immediately adjacent to vessels without concerns of incomplete ablation because of the continual flow of warm blood.

REFERENCES

DISCUSSION

Dr. Arthur W. Boddie, Jr. (Chicago, Ill.). My concern is whether a tumor is likely to behave in the same fashion as normal tissue. In your study, necrosis was the primary mode of cell death, suggesting that rapid cooling occurred during cryoablation. With a large tumor and a large vessel at the periphery, there may be a zone around the vessel in which portions of the tumors undergo somewhat slower decreases in temperature. If this is the case, some investigators have reported that apoptosis rather than necrosis may be the primary mode of cell death, and tumor cells may be somewhat more resistant to apoptosis than necrosis based on either mutations in p53 or up-regulation of the BCL-2 gene. Also, some studies of hypothermia in animals have indicated that cold may induce heat shock proteins, which are now seen to protect cells not only from heat but also from a variety of other stress states. Conceivably, this might also affect the ability to kill tumor cells in this vicinity. Can you comment on these two issues?

Dr. Mahvi. This study did evaluate only normal hepatic parenchyma. The two tumor types that we most commonly evaluate (hepatic primaries and metastatic colorectal cancers) may behave differently. We think that these results are applicable to hepatocellular carcinoma, which has less fibrous tissue than metastatic colorectal carcinoma.

Clinically we observe freezing right up to vessel walls by ultrasound in both tumor types. We performed this study to prove that the clinical methodology used made sense.

As far as apoptosis versus freezing due to ice crystal formation in cells, no clinical data are available to answer that question. The interesting thing about these cryolesions is that the demarcation between dead and alive tissue is very sharp. The amount of freezing at the periphery of the lesion, although the rate of freeze is much slower, does seem to correlate well with cell death.

We have looked at induction of a heat shock protein (HSP-27) in an in vitro model, and at least the induction of HSP-27 by various noxious agents is correlated with this HSP-27 expression. Up-regulation of HSP-27 does seem to protect cells. At least in this system, if we can get cell at the edge of a cryolesion, I don’t think we give the cells enough chance to recover because freezing is a little bit too rapid.

Dr. David M. Ota (Columbia, Mo.). Metastatic tumors are often adjacent to major vessels. This can be seen on CT scan. Did you use ultrasonography when you placed your probe, and did you place the probe next to a large vessel? It seems that your probe was in the periphery of the liver. Were you able to freeze right up to a major vessel instead of small vessels?

Dr. Mahvi. We did attempt to place the probes in a peripheral location. The largest vessel that we examined was 4.5 mm, which is a relatively large vessel in a pig. Clinically, when I place these probes I try to skew toward major vessels.

One of the indications for cryosurgical ablation is the presence of tumors right next to hepatic veins. We try to place the cryoprobe right next to the hepatic vein and let the bulk of the ice ball proceed away from the vessel. That gives us a better chance of ablating that tumor than resecting it, which can be difficult at the hepatic vein confluence.

Dr. Richard A. Prinz (Chicago, Ill.). Dr. Edgar Staren in my department has evaluated the one to two freeze/thaw cycle. His data are much different from yours. They indicate that a second freeze is very important. His model uses tumor in the liver, which is an important difference, and also he has longer follow-up. Our problem in dealing with patients is not that we leave the operating room thinking we have left tumor behind, it is that we don’t know we have left viable tumor there. A longer follow-up period is required to evaluate this problem. I remain unconvinced that we should switch to a single-freeze cycle.

Dr. Mahvi. I would respond by saying that no data suggest that it is better to use multiple freeze cycles. There are advantages to using multiple freeze cycles; you get a larger cryolesion, which is needed to cover the tumor, depending on the tumor size. However, the published experimental data with regard to different kill rates for different numbers of cryolesions are not convincing.

Dr. Jack Pickleman (Maywood, Ill.). Do you have any clinical data to convince us that this technique is preferable?

Dr. Mahvi. We were concerned that this technique was going to be applied clinically without sufficient experimental data to show that it worked. I think experimental surgery is an important tool to try to answer these questions before the procedures are widely adopted in clinical practice.

ANNOUNCEMENT

Effective October 1, 1997, please send all manuscripts and other submissions for Surgery to the following address:

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