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The Apoptotic Response of Liver and Colorectal Liver Metastases to Focal Hyperthermic Injury

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Abstract. *Background: Ablation of liver tumours by focal hyperthermia causes tissue injury not only by direct effects, but also by progressive tissue damage following the initial heat application. The mechanism by which this progressive injury occurs remains undefined. Stimulation of apoptosis following the initial heat stimulus may be involved in this progression. The role of apoptosis in the subsequent progression of focal hyperthermia injury was investigated in liver and colorectal liver metastases in a murine model. Materials and Methods: Focal hyperthermia produced by laser (Nd-YAG - wavelength 1064 nm) was applied to the liver and colorectal liver metastases in CBA mice (2 Watts for 50 seconds). The animals were killed at 0, 12, 24, 48, 72, 120 and 168 hours after the application of focal hyperthermia. Haematoxylin and eosin staining and immunohistochemistry were performed on paraffin sections to assess the extent of tissue necrosis. Apoptosis was examined by assessment of DNA fragmentation using terminal deoxynucleotidyl transferase d-uridine triphosphate nick end labelling (TUNEL) and activated Caspase 3 immunostaining. The sequence of the apoptotic response was determined at the treated tissue margins and compared to untreated liver and tumour. Results: Focal hyperthermia produced progressive tissue injury in both liver and colorectal liver metastases. TUNEL labelling was less specific than activated Caspase 3 in detecting apoptotic cells. Apoptosis was detected and peaked at 12 hours following therapy based on activated Caspase 3 staining, before returning to baseline at 72 hours. In tumour tissue, the apoptotic response was more sustained, peaking at 24 hours before returning to baseline levels by 96 hours following therapy. Conclusion: Increased apoptosis occurs following the application of focal hyperthermia to normal liver and colorectal metastases. The apoptotic response is more sustained in tumour tissue and contributes to the progressive injury that is evident after the initial heat stimulus.*

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Focal hyperthermia, produced by either laser, radiofrequency or microwave therapy, has been clinically applied for local ablation of liver tumours (1). Clinical and experimental studies suggest that the area of tissue injury following focal hyperthermia increases after cessation of the initial heat stimulus (2-6). Heat activation of apoptosis may be involved in the progression of injury and possibly is an important determinant of the completeness of tumour eradication (7,8). Few studies, however, define the role of apoptosis in liver and tumour tissue destruction following focal hyperthermia application.

This study investigated the role of apoptosis in the progression of injury following the application of focal hyperthermia by laser to liver and colorectal liver metastases in a murine model.

Materials and Methods

Animals. Male inbred CBA strain mice, 6-8 weeks of age, were used in these experiments. The animals were housed in standard cages with access to irradiated food and water *ad libidum* and exposed to a 12-hour light/dark cycle. Animals with and without liver metastases were studied. All procedures were performed according to the guidelines of the Austin Hospital Animal Ethics Committee, Australia.

Liver metastases model. A dimethyl hydrazine (DMH) derived primary murine colon cancer cell line (MoCR) was used for the induction of colorectal liver metastases by methods previously described (9). The histology, vasculature and growth kinetics of these tumours closely resemble human colorectal liver metastases (9-11).

Preparation of cell suspension and induction of metastases. A cell suspension (1 x 10^6 cells / ml) was prepared by standard techniques

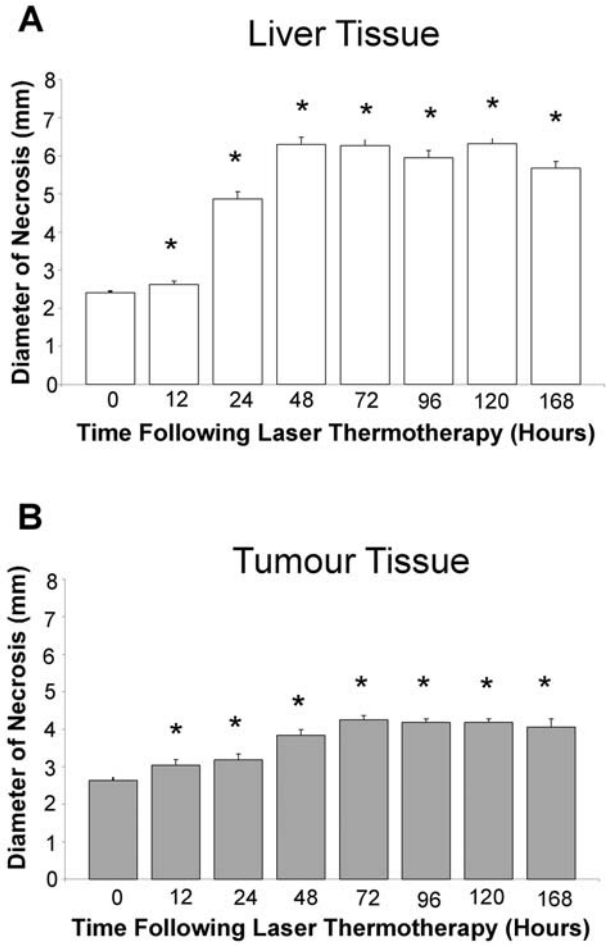

Figure 1. *(A) Diameter of necrosis in liver following laser therapy. The diameter of necrosis by 12 hours was significantly greater (p=0.029) than the immediate injury. A further significant increase in necrosis occurred between 12 hours and 24 hours (p <0.001) and 24 hours and 48 hours (p<0.001). (B) Tumour tissue necrosis following laser application. A statistically significant increase in necrosis is noted at 12 hours (p=0.029) and beyond (p<0.001) compared to the initial injury at 0 hours. Further significant increases occurred between 12 hours and 48 hours (p=0.004) and 48 and 72 hours (p=0.041). (Mann Whitney U-test) (*p<0.05 compared to the initial injury).*

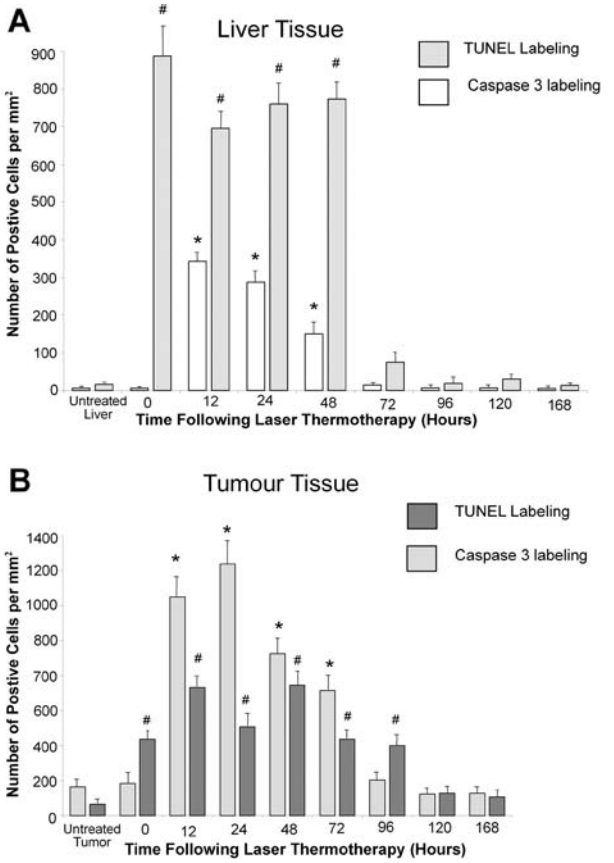

Figure 2. *(A) Apoptotic response based on TUNEL and Caspase 3 labelling compared to untreated liver. Dramatic increase in TUNEL staining is noted immediately following focal hyperthermia application and returns to baseline at 72 hours (# p<0.001). In contrast, Caspase 3 labelling increases and peaks at 12 hours following therapy returning baseline levels at 72 hours (* p<0.001). (B) In tumour tissue there is similarly an increase in TUNEL labelling immediately following focal hyperthermia application which returns to baseline at 120 hours (# p<0.001). Caspase 3 labelling increases and peaks at 24 hours following therapy returning to baseline levels at 96 hours (* p<0.001). (Mann Whitney U-test)*

(9). Mice were induced with metastases by intrasplenic injection of 50,000 cells over one minute, followed by splenectomy and wound closure. Twenty-one days after tumour induction, animals bearing fully established liver metastases were used for subsequent study.

Focal hyperthermia. A Neodymium Yttrium-Aluminium-Garnet (Nd:YAG-wavelength of 1064 nm) laser (Dornier medilas fibertom 4100 Medizintechnik GmbH, Munchen, Germany) was used as a focal heat source. Following anaesthesia, a bilateral subcostal incision was used to fully expose the liver. A bare tipped optical quartz fibre of 400 μm diameter was used to deliver laser energy. This was applied to either the liver of normal animals or tumour tissue in animals with colorectal liver metastases. Intraparenchymal tumours (7 to 8 mm diameter), which were comparable in size and location, were chosen for therapy. The liver was removed following the completion of procedures for assessment of initial injury. In all other animals, the abdomen was closed and the animals were killed at predetermined time points.

Preliminary experiments determined the power and exposure time for the application of laser that consistently produced a region of immediate coagulation 2 to 3 mm in diameter. Tissue temperature parameters 2 to 3 mm from the site of fibre insertion ranged from 39˚C to 41˚C and were similar in tumour and liver tissue immediately upon completion of therapy. Tissue temperatures returned to baseline levels within two minutes of the completion of therapy. The settings selected were to cause incomplete tumour necrosis and allow assessment of tumour response in isolation from the liver response.

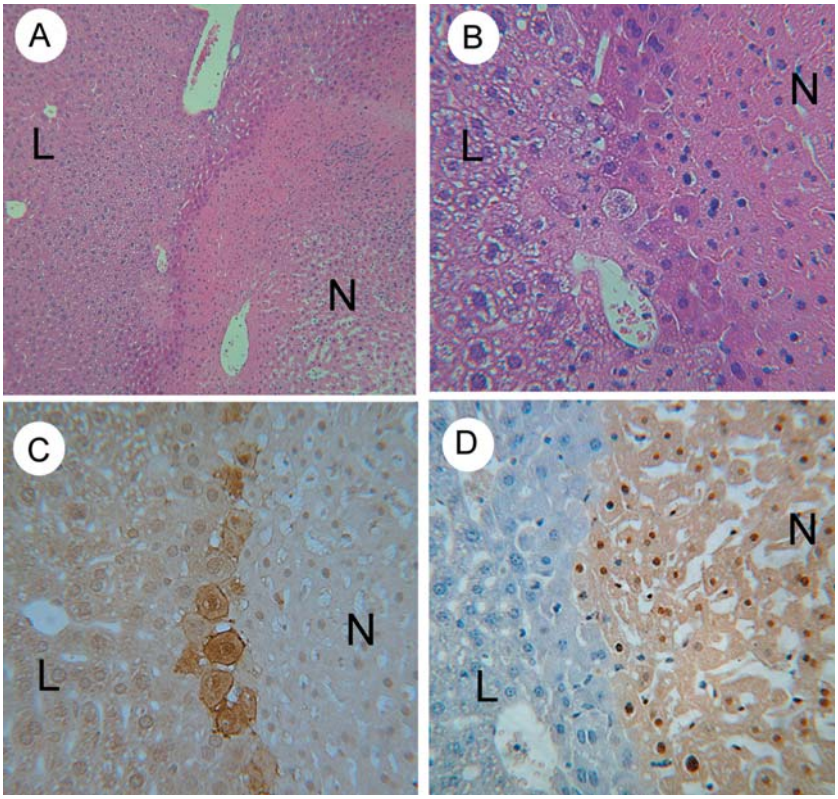

Figure 3. *(A) Tissue injury and apoptosis in normal liver 12 hours following therapy. Regions of necrosis (N) can be distinguished from unaffected liver (L) (Magnification x 150). (B) Morphologically apoptotic hepatocytes with intensely eosinophilic staining cytoplasm and pyknotic nuclei are evident at high magnification (Magnification x 550). (C) Caspase 3 immunostaining clearly identified morphologically apoptotic cells (Magnification x 550). (D) TUNEL staining of cells at the interface between necrotic tissue and liver. Both morphologically normal and apoptotic cells are positively stained (Magnification x 550).*

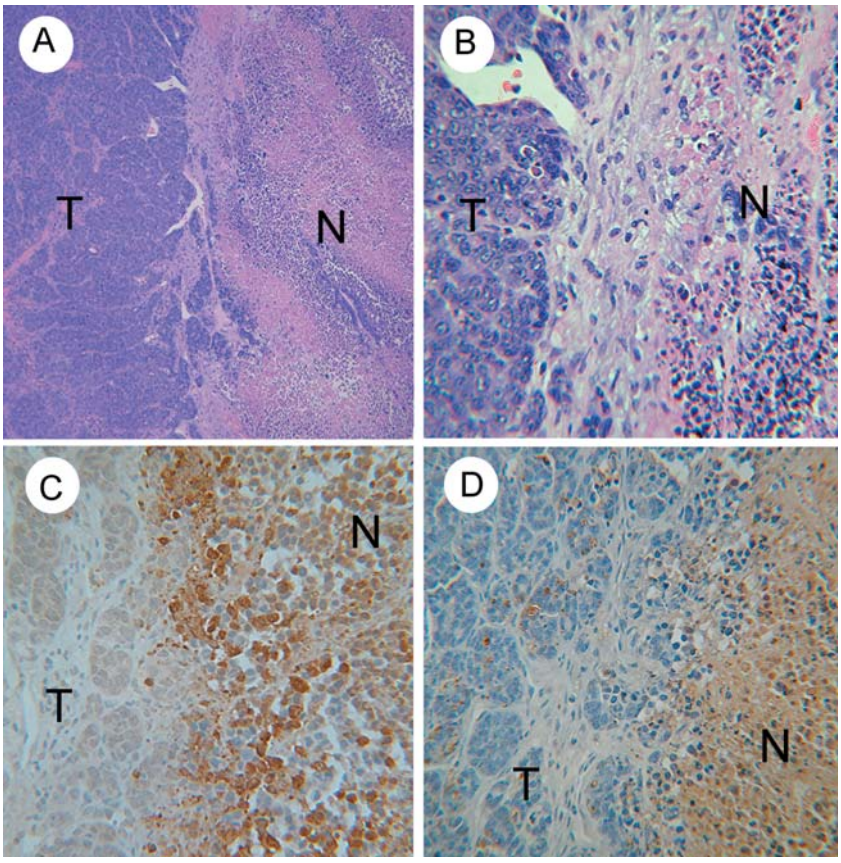

Figure 4. *(A) Tissue injury and apoptosis in tumours 24 hours following therapy. Regions of necrosis (N) can be distinguished from tumour (L) (Magnification x 150). (B) Morphological features of apoptosis at high magnification are not as apparent as normal liver (Magnification x 550). (C) Caspase 3 immunostaining clearly identifying apoptotic cells (Magnification x 550). (D) TUNEL staining at the interface between necrotic tissue and tumour tissue is less prominent than the Caspase 3 staining (Magnification x 550)*

In a group of control animals, the laser fibre was inserted into normal liver or tumour without activation. The animals were then killed at matched time-points to the focal hyperthermia treatment groups.

Experimental groups. Animal numbers were based on preliminary studies, estimating a minimum of 10 animals per study group to detect a 10 to 15 per cent change in apoptosis at the different time points with a power of 0.8 and $p<0.05$.
Study 1 – Focal hyperthermia induced apoptosis in normal liver: The effect of focal hyperthermia on apoptosis in normal liver was assessed at predetermined time points (0, 12, 24, 48, 72, 96, 120 and 168 hours) in separate groups (n=10) of animals after laser application. Matched controls were studied at similar time points.
Study 2 – Focal hyperthermia induced apoptosis in colorectal liver metastases: The effect of focal hyperthermia on apoptosis in colorectal liver metastases was assessed at similar time points as study 1, namely 0, 12, 24, 48, 72, 96, 120 and 168 hours in separate animal groups (n=10). Control animals with tumours were also studied at the same time points following fibre insertion without activation.

Assessment

Histological assessment of tissue necrosis: Tissue that was removed for study was fixed in 10% formalin for 24 hours. It was then cut perpendicular to the point of laser fibre insertion and embedded in paraffin. Relevant tissue was sectioned at 4 μm thickness and stained with haematoxylin and eosin (H&E). Histological assessment was performed using an Olympus light microscope (BHT, Olympus, Tokyo, Japan). The diameter of necrosis was determined and recorded at the various time points in a blinded manner.
TUNEL staining: Tissue embedded in paraffin was sectioned to 4-μm slices and deparaffinized. DNA strand breaks were detected by terminal deoxynucleotidyl transferase d-uridine triphosphate nick end labelling (TUNEL) using an *in situ* detection kit (R&D Systems, NSW, Australia). Endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 5 minutes at room temperature. Specimens were then pre-treated with Proteinase K (20 μg/ml) for 30 minutes at 37˚C in phosphate-buffered saline (PBS) (pH 7.4). TUNEL labelling was performed according to the manufacturer's directions. Negative sections were made for all specimens. Tissue sections were incubated for 8 minutes in diaminobenzidine (DAB) solution. Nuclei were counterstained with methyl green, mounted in DePeX (Gurr®, BDH Laboratories, Poole, UK) and cover-slipped for optical clarity.

Activated Caspase 3 staining: Tissue sections were prepared as described for TUNEL. A non-biotin labelling kit was used for staining (Histomouse Max, Zymed, CA, USA). Antigen retrieval was performed by incubation of sections in citrate pH 6.0 (99˚C to 100˚C) for 10 minutes, followed by cooling at room temperature for 25 minutes. Endogenous peroxidase activity was inhibited by 3% H_2O_2 in methanol for 10 minutes at room temperature. Tissue sections were incubated with activated Caspase 3 (R&D Systems, NSW, Australia) at room temperature for 90 minutes (1:100). Labelling was achieved using a strepdavidin HRP complex. Positive cells were identified by incubation with DAB solution for 10 minutes. The same procedures were carried out on negative controls, replacing the primary antibody with PBS.

Quantitative apoptotic index: Apoptosis at each time point was estimated by counting positively-stained cells. The morphological characteristics of positively-stained cells were referenced to equivalent H&E-stained sections. TUNEL-positive cells were identified by distinct brown nuclei, whereas activated Caspase 3-positive cells contained brown-stained cytoplasm. Microphotographs of tissue sections were taken at a magnification of x 150. Random regions measuring 200 μm x 200 μm were selected at the outer margin of the treated tissue using an image analysis program (Image-Pro Plus Version 4.5, Media Cybernetics, CA, USA). Cells were counted in a blinded manner and the mean counts were expressed as the number of positive cells per mm^2. A minimum of 10 regions were examined for each specimen. Untreated liver and tumour tissue sections were similarly randomly assessed.

Statistical analysis: Statistically significant differences in mean(S.E.) between the different animal groups were compared by the Mann Whitney *U*-test (SPSS®,Chicago, Illinois, USA). A *p* value of less than 0.05 was considered statistically significant.

Results

Study 1 – Focal hyperthermia induced apoptosis in liver. Histological changes indicative of tissue necrosis were poorly defined immediately following thermal application. Clear demarcation of tissue necrosis was apparent in liver by 12 hours following injury. The diameter of coagulated tissue at 12 hours following focal hyperthermia was significantly greater than the initial injury (2.4(±0.04)mm *vs.* 2.6(±0.1)mm; $p=0.029$) (Figure 1). Further statistically significant increases occurred between 12 hours and 24 hours (2.6(±0.1)mm *vs.* 4.9(±0.2)mm; $p<0.001$) and 24 and 48 hours (4.9(±0.2)mm *vs.* 6.3(±0.2)mm; $p<0.001$).

TUNEL-positive cells were seen infrequently in normal liver tissue (16(±5.5) cells/mm^2) (Figure 2). There was a dramatic increase and peak in TUNEL staining immediately after laser application (888(±88.9) cells/mm^2) ($p<0.001$). TUNEL-positive cells extended from the region surrounding the site of heat application to normal liver. The majority of positively-stained cells at all time points were either morphologically normal or undergoing necrosis (Figure 3). TUNEL staining remained significantly elevated at 48 hours (773(±52.3) cells/mm^2) following laser injury compared to untreated liver tissue ($p<0.001$) (Figure 2).

Activated Caspase 3 labelling was more specific in delineating morphologically apoptotic cells. These cells exhibited pyknotic nuclei and densely eosinophilic cytoplasmic staining (Figure 3). Staining was equivalent to baseline levels immediately following injury (5(±3.4) cells/mm^2 *vs.* 6(±2.9) cells/mm^2; $p=0.931$). A significant increase and peak in activated Caspase 3 staining was noted 12 hours following focal hyperthermia treatment compared to untreated liver (342(±21.9) cells/mm^2 *vs.* 5(±3.4) cells/mm^2; $p<0.001$) (Figure 2). Positively-stained cells were confined to the periphery of the treatment zone. Activated Caspase 3 staining

remained significantly elevated at 48 hours following laser injury compared to untreated liver tissue (150(±35.1) cells/mm^2 *vs.* 5(±3.4) cells/mm^2; $p<0.001$) (Figure 2).

Laser fibre application without activation in control animals caused no increase in apoptosis immediately following treatment based on TUNEL or Caspase 3 staining. A significant increase in apoptosis was, however, noted at 12 hours following injury, which returned to baseline by 24 hours by both methods.

Study 2 – Focal hyperthermia induced apoptosis in colorectal liver metastases. Tissue necrosis was poorly defined both macroscopically and on histology immediately after the application of focal hyperthermia. The tissue necrosis was limited adjacent to the site of fibre application. Demarcation of tissue injury in tumour tissue was apparent by 12 hours following injury (Figure 4). This was less well defined when compared to the injury in normal liver. The diameter of coagulated tissue at 12 hours following focal hyperthermia application was significantly greater than the initial injury based on H&E histology(2.6(±0.1)mm *vs.* 3.0(±0.1)mm; $p=0.025$)(Figure 1). Further significant increases were noted between 12 hours and 48 hours (3.0(±0.1)mm *vs.* 3.8(±0.1)mm; $p=0.004$) and 48 and 72 hours (3.8(±0.1)mm *vs.* 4.3(±0.1)mm; $p=0.041$). There was no statistically significant increase in the diameter of necrosis beyond 72 hours.

Apoptotic cells were present in untreated tumours in high concentration surrounding areas of spontaneous tumour necrosis. Morphological features of apoptosis were difficult to identify on histology of tumour tissue. There was a significant increase in TUNEL-positive cells in tumour tissue surrounding the application site immediately after laser application compared to untreated tumours (437(±36.0) cells/mm^2 *vs.* 66(±13.2) cells/mm^2; $p<0.001$) (Figure 2). The majority of these cells were not morphologically apoptotic. TUNEL staining at the periphery of treated regions remained significantly elevated at 96 hours following treatment compared to untreated tumours (400 (±60.0) cells/mm^2 *vs.* 66(±13.2) cells/mm^2; $p<0.001$), returning to baseline by 120 hours.

Similar to TUNEL staining, activated Caspase 3-positive cells were concentrated around regions of spontaneous necrosis in untreated tumour tissue. A significant increase in activated Caspase 3 staining was noted at the periphery of treated regions by 12 hours following focal hyperthermia compared to untreated tumour (1250(±112.1) cells/mm^2 *vs.* 165(±28.2) cells/mm^2; $p<0.001$). The peak response occurred at 24 hours following treatment (1438(±130.9) cells/mm2), before a gradual return to baseline levels by 96 hours following treatment.

In control animals, no increase in apoptosis was detected in tumour tissue immediately following application of a laser fibre without activation by either staining method. A significant increase in apoptosis was, however, noted in tumour tissue by both methods at 12 hours following injury, which returned to baseline levels by 24 hours.

Discussion

There is a growing emphasis on minimally invasive percutaneous techniques for the treatment of liver tumours. Focal hyperthermia by laser or radiofrequency are currently the favoured *in situ* ablative techniques in selected patients with liver tumours (12,13). There is, however, a significant rate of local recurrence following application of focal hyperthermia ranging from 1 to 10% (1). The major limitation of tumour ablation by focal hyperthermia is the extent of tissue injury that can be achieved. Methods aimed at increasing the size of ablation have traditionally focused on modification of the fibre design and temperature control to allow greater power and energy delivery to tissues without charring. In addition, inflow vascular occlusion combined with focal hyperthermia has been investigated as a potential method to minimize heat dissipation and ensure complete tumour destruction (1).

Several recent studies demonstrated a progression in tissue damage following the initial focal hyperthermia injury that results in an increase of the ablative site (2-6). The underlying mechanism of this increase in tissue damage is undefined and almost certainly involves complex and multifactorial processes. Stimulation of apoptosis by hyperthermia is a major factor involved in this progression of tissue injury. *In vitro* studies show increased apoptosis in cell culture in a temperature-dependent manner (7,8). The effect of focal hyperthermia on apoptosis is, however, poorly defined.

Recently, Ohono *et al.* (14) demonstrated the finding of increased apoptosis at the periphery of the application zone following microwave ablation of liver in rats. The area of liver injury increased to a peak at 12 hours following microwave coagulation. TUNEL staining showed increased DNA fragmentation 2 hours following injury, which remained elevated for 24 hours post microwave treatment. Caspase 3 activity in liver tissue homogenates peaked at 2 hours following injury, promptly returning to baseline thereafter.

Apoptosis is known to occur following exposure of cells to various factors including heat, hypoxia and radiation. In apoptotic cells there is nuclear damage with fragmentation of DNA (15). This results from the activation of intercellular signalling pathways including the activation of various kinases and cysteine-directed asparagines proteases, known as caspases. Caspase 3 is the downstream effector caspase that is essential for DNA fragmentation.

Tissue temperatures ranging from 42˚C to 45˚C for 30 to 60 minutes during classical hyperthermia causes inhibition of cellular enzymes and cell damage largely through the

process of apoptosis (3,16-19). Local ablation by focal hyperthermia generally achieves temperatures ranging from 60˚C to 100˚C adjacent to the site of fibre insertion, causing immediate coagulative cell death (12). The apoptotic response following focal hyperthermia is expected to predominate at the periphery of treatment regions, where cells are exposed to temperatures below 60˚C.

Focal hyperthermia is also known to damage endothelial cells and alter blood flow, leading to small vessel thrombosis following the cessation of the heat stimulus (20-22). Tissue hypoxemia at the periphery of the treated lesions rather than hyperthermia *per se* may stimulate apoptosis. In addition, hyperthermia stimulation of Kupffer at temperatures not exceeding 43˚C may induce apoptosis through direct and indirect pathways (23-25).

A progression of lethal tissue damage and stimulation of apoptosis following focal hyperthermia application was demonstrated by immunohistochemistry in both liver and colorectal liver metastases in this study. An increase in tissue damage was demonstrated in tumours for 72 hours following injury. An elevated apoptotic response was similarly demonstrated for 72 hours following injury. In normal liver, progressive tissue injury occurred for 48 hours following heat application, with a similar response in apoptosis. In sublethally damaged tumour tissue, the progression of injury is likely to be more limited than in liver due to rapid in-growth of surrounding tumour cells into the damaged region with time. This may partly explain the lower absolute increase in tumour damage following focal hyperthermia compared to liver injury, despite a more prolonged apoptotic response.

An increased apoptotic response to focal hyperthermia was demonstrated in liver and colorectal liver metastases by TUNEL and Caspase 3 immunohistochemistry. The baseline apoptotic rate in tumours was greater than liver, as expected, particularly adjacent to areas of spontaneous necrosis. There was a dramatic increase in the number of TUNEL-positive cells in both tumour and normal liver immediately following laser application. The majority of positive cells were morphologically normal and at this early stages of necrosis. The high rate of TUNEL-positive cells immediately after the application of focal hyperthermia is probably related to laser-induced DNA strand breaks (26). These TUNEL-positive cells are in the early phases of necrosis rather than apoptosis, with no morphological features to support the latter. They were not detected by Caspase 3 staining, which appeared to identify morphologically apoptotic cells at all time points. The apoptotic response in liver was more prolonged in this study than reported by Ohono *et al.* (14), extending beyond 24 hours following therapy. This can partly be explained by differences in assessment, with immunohistochemical assessment of the periphery of lesions expected to detect smaller differences than biochemical assays

The increased apoptotic response at the periphery of treated areas following hyperthermia may be related to one or more known stimuli of cellular apoptosis. The periphery of treated lesions was exposed to temperatures below 41˚C, which is known to induce apoptosis *in vitro* and possibly explains some of the findings in this study. The continued apoptotic response beyond 24 hours is, however, unlikely to be related to thermal effects. It possibly relates to thermal stimulation of other process that include local cytokine release, tissue ischemia and reperfusion injury. Importantly, the application of a laser fibre to liver and tumour without activation induced only a brief apoptotic response. This suggests that the stimulation of apoptosis by focal hyperthermia is essentially a heat-specific response.

This study clearly demonstrated that stimulation of apoptosis is involved in the progression of injury following focal hyperthermia in liver and colorectal metastases. The combination of focal hyperthermia with therapies that induce apoptosis may potentially have synergistic effects. Such a method may ensure more complete tumour destruction, reduce local recurrences and potentially improve survival and is worthy of further investigation.

References

1 Curley SA, Cusack JC Jr, Tanabe KK and Ellis LM: Advances in the treatment of liver tumors. Curr Probl Surg *39*: 449-571, 2002.

2 Nikfarjam M, Malcontenti-Wilson C and Christophi C: Focal hyperthermia produces progressive tumor necrosis independent of the initial thermal effects. J Gastrointest Surg. 2005 (in press).

3 Matthewson K, Coleridge-Smith P, O'Sullivan JP, Northfield TC and Bown SG: Biological effects of intrahepatic neodymium:yttrium-aluminum-garnet laser photocoagulation in rats. Gastroenterology *93*: 550-7, 1987.

4 Matsumoto R, Selig AM, Colucci VM and Jolesz FA: Interstitial Nd:YAG laser ablation in normal rabbit liver: trial to maximize the size of laser-induced lesions. Lasers Surg Med *12*: 650-8, 1992.

5 Sweetland HM, Wyman A and Rogers K: Evaluation of the effect on the normal liver of interstitial laser hyperethermia using artificial sapphire probes. Lasers Med Sci *8*: 99-105, 1993.

6 Wiersinga WJ, Jansen MC, Straatsburg IH, Davids PH, Klaase JM, Gouma DJ and van Gulik TM: Lesion progression with time and the effect of vascular occlusion following radiofrequency ablation of the liver. Br J Surg *90*: 306-12, 2003.

7 Barry MA, Behnke CA and Eastman A: Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. Biochem Pharmacol *40*: 2353-62, 1990.

8 Benndorf R and Bielka H: Cellular stress response: stress proteins--physiology and implications for cancer. Rec Res Cancer Res *143*: 129-44, 1997.

9 Kuruppu D, Christophi C, Bertram JF and O'Brien PE: Characterization of an animal model of hepatic metastasis. J Gastroenterol Hepatol *11*: 26-32, 1996.

10 Kuruppu D, Christophi C and O'Brien PE: Microvascular architecture of hepatic metastases in a mouse model. HPB Surg *10*: 149-57; discussion 158, 1997.
11 Nikfarjam M, Muralidharan V, Malcontenti-Wilson C and Christophi C: Scanning electron microscopy study of the blood supply of human colorectal liver metastases. Eur J Surg Oncol *29*: 856-61, 2003.
12 Nikfarjam M and Christophi C: Interstitial laser thermotherapy for liver tumours. Br J Surg *90*: 1033-47, 2003.
13 Erce C and Parks RW: Interstitial ablative techniques for hepatic tumours. Br J Surg *90*: 272-89, 2003.
14 Ohno T, Kawano K, Sasaki A, Aramaki M, Yoshida T and Kitano S: Expansion of an ablated site and induction of apoptosis after microwave coagulation therapy in rat liver. J Hepatobiliary Pancreat Surg *8*: 360-6, 2001.
15 Reed JC: Mechanisms of apoptosis. Am J Pathol *157*: 1415-30, 2000.
16 Welch AJ, Motamedi M, Rastegar S, LeCarpentier GL and Jansen D: Laser thermal ablation. Photochem Photobiol *53*: 815-23, 1991.
17 Thomsen S: Pathologic analysis of photothermal and photomechanical effects of laser-tissue interactions. Photochem Photobiol *53*: 825-35, 1991.
18 Overgaard J and Suit HD: Time-temperature relationship in hyperthermic treatment of malignant and normal tissue *in vivo*. Cancer Res *39*: 3248-53, 1979.
19 van Hillegersberg R, de Witte MT, Kort WJ and Terpstra OT: Water-jet-cooled Nd:YAG laser coagulation of experimental liver metastases: correlation between ultrasonography and histology. Lasers Surg Med *13*: 332-43, 1993.
20 Fajardo LF, Egbert B, Marmor J and Hahn GM: Effects of hyperthermia in a malignant tumor. Cancer *45*: 613-23, 1980.
21 Emami B, Nussbaum GH, TenHaken RK and Hughes WL: Physiological effects of hyperthermia: response of capillary blood flow and structure to local tumor heating. Radiology *137*: 805-9, 1980.
22 Kelleher DK, Engel T and Vaupel PW: Changes in microregional perfusion, oxygenation, ATP and lactate distribution in subcutaneous rat tumours upon water-filtered IR-A hyperthermia. Int J Hyperthermia *11*: 241-55, 1995.
23 Yoshioka H, Koga S, Maeta M, Shimizu N, Hamazoe R and Murakami A: The influence of hyperthermia *in vitro* on the functions of peritoneal macrophages in mice. Jpn J Surg *20*: 119-22, 1990.
24 Andreesen R, Osterholz J, Schulz A and Lohr GW: Enhancement of spontaneous and lymphokine activated human macrophage cytotoxicity by hyperthermia. Blut *47*: 225-9, 1983.
25 Dickson JA: Hyperthermia in the treatment of cancer. Lancet *1*: 202-5, 1979.
26 Muralidharan V and Christophi C: Interstitial laser thermotherapy in the treatment of colorectal liver metastases. J Surg Oncol *76*: 73-81, 2001.

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