ULTRASOUND TUMOR SIZE ASSESSMENT, HISTOLOGY AND SERUM ENZYME ANALYSIS IN A RAT MODEL OF COLORECTAL LIVER CANCER

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Abstract

During tumour development, tissue necrosis appears as a natural phenomenon directly associated with an increase in tumour size. The aim of this study was to assess the use of ultrasound (US) for predicting natural tumour necrosis in a rat liver implant model of colorectal cancer. To achieve this goal, we sought to establish a correlation between US-measured tumour volume, serum enzyme levels and histopathological findings, in particular regarding necrosis phenomena in the liver implants.

Under US guidance, CC531 colorectal cancer cells were injected into the left liver lobe of WAG/RijHsd rats. Twenty-eight days after cell inoculation, the volume of tumours was measured by US, and rats were sacrificed to obtain samples of tumour tissue as well as blood serum. In haematoxylin-eosin-stained tumour samples, the percentage of the tumour that was necrotic was estimated. The association between tumour necrosis percentage and US-measured tumour volume was assessed by univariate logistic regression analysis, and the linear regression equation was obtained.

Serum enzyme levels did not differ significantly between tumour-bearing and tumour-free rats. Tumour implants appeared as well-defined hyperechoic regions, with a mean volume of 0.61 ± 0.39 ml and a tumour necrosis percentage of 8.6 ± 7.7%. The linear regression analysis showed that there was a very strong relationship (“Person correlation coefficient” r=0.911) between US-measured tumour volume and tumour necrosis percentage, and the regression equation was: tumour necrosis percentage = 21 x US-measured tumour volume [in ml] - 3.1.

Our study demonstrates US to be a useful tool for animal-based trials. Tumours inside the liver can be observed by US (when ranging in size from 0.24 to 1.37 ml),

and moreover, US-measured tumour volume on day 28 can be used to estimate tumour necrosis occurring as natural evolution of tumour implants.

Keywords: orthotopic tumour model, intrahepatic injection, US guided injection & tumour volume assessment.

Introduction

Cancer is the second leading cause of death in the European Union (EU). The Statistical Office of the EU (Eurostat) estimated that cancer was the cause of almost 1.3 million deaths in 2013, colorectal cancer (CRC) being responsible in 12% of these (Eurostat 2016). About 25% of patients with CRC have synchronous liver metastases at the time of diagnosis or during treatment (Garden et al. 2006), and approximately 40-60% will develop metachronous liver metastases in the 5 years following diagnosis (Garden et al. 2006; Manfredi et al. 2006).

Despite only 10 to 20% of patients with colorectal cancer liver metastases (CRCLM) being eligible for surgical treatment (Poston et al. 2008; Van den Eynde and Hendlisz 2009), hepatic resection remains the gold standard treatment for CRCLM. For patients in whom surgical resection or ablative therapies, such as radiofrequency ablation, are not appropriate, the preferred treatment is systemic therapy with chemotherapeutic combination therapy (e.g., FOLFIRI or FOLFOX) (Hess et al. 2010; Zuckerman and Clark 2008). Under FOLFIRI chemotherapy, 33% of patients with unresectable CRCLM were found to become suitable for hepatectomy or metastasectomy (Barone et al. 2007); and the FOLFOX regimen also results in a reduction of unresectable CRCLM, 16 to 40% of patients becoming candidates for resection after this treatment (Alberts et al. 2005; Giacchetti et al. 1999). Despite these good results, these therapies are often not well tolerated, due to their side effects and their low specificity for tissue or tumour cells. Therefore, it is increasingly important to use and develop experimental models that enable us to study the selective administration of treatments.

Though many experimental studies are carried out in mice because of the lower associated cost and availability of nude animals to host human-derived tumours,

74 they are so small that intrarterial therapies cannot be tested. In contrast, tumour
75 models in syngeneic rats allow implementation of many of the same surgical
76 approaches used in humans and these animals have an intact immune system
77 which is quite important in tumour biology.

78 Such experimental models should be designed bearing in mind the 3Rs principle
79 (European Union 2010), seeking a “reduction” in the number of animals,
80 “refinement” of surgical procedures (minimising the associated pain and
81 invasiveness) and the “replacement” of animal models with non-animal alternatives.
82 Variability in the results obtained is considered one of the main obstacles to using
83 small numbers of animals. Using a model of direct implantation of cancer cells into
84 the liver parenchyma results in more homogeneous and well-defined tumour foci in
85 predictable locations. If the treatment tested does not involve the biological
86 processes of metastases, this approach is justified at least in the first steps of
87 research.

88 Studies with syngeneic tumour cells, though very effective, still fail to produce
89 tumours in a substantial percentage of animals (Adwan et al. 2017; Kim et al. 2009;
90 Robertson et al. 2008). Detecting which animals do not bear tumours with a non-
91 invasive tool would allow their exclusion from a study, reducing costs and effort. In
92 relation to this, US with a high frequency probe can be considered an affordable
93 tool for any experimental researcher.

94 Another benefit may also be obtained from studying animals with non-invasive
95 techniques, namely, the detection and quantification of the necrosis developing
96 inside the tumour. At least in the animal model used by our group (described below),
97 tumour necrosis can be observed as a natural phenomenon directly related to the
98 increase in tumour size. Since some treatments also induce tumour necrosis, it is

very important to be able to distinguish between naturally-occurring and treatment-induced necrosis. As it has been established that tumour necrosis is closely correlated with tumour volume (Baker et al. 19900; Milross et al. 1997), we propose that volume measured by US should allow us to estimate the percentage of natural necrosis occurring in the tumour.

In this study, we measured tumour volume using US and quantified the area of necrosis histopathologically in an experimental model of liver cancer. Our aim was to obtain an equation that would allow us to estimate the necrosis percentage in a tumour as a consequence of its natural growth and to distinguish this from tumour necrosis associated with treatment.

Materials and methods

All the procedures used in this study were performed in strict accordance with the recommendations of current national legislation on experiments involving animals and/or biological agents. The protocols were approved by the Ethics Committee on Animal Experimentation (CEEA) (ref. number: CEBA/140/P02-01/2010/ALONSO VARONA and M20/CEEA/407/2015/HERRERO DE LA PARTE) and Ethics Committee for Research involving Biological Agents and Genetically Modified Organisms (CEIAB) (ref. number: M30/2016/024/HERRERO DE LA PARTE) of the University of the Basque Country (UPV/EHU).

Cell culture and experimental model

The study was conducted using the CC531 tumour cell line. This cell line is moderately differentiated and was originally obtained from CRC induced by 1,2-dimetylhydrazine in WAG/RijHsd rats (also called WAG/Rij or WAG/RijCrl). CC531

cells were purchased from Cell Line Services (Eppelheim, Baden-Württemberg, Germany) in passage 22 and mycoplasma negative (in specific PCR), and after amplification, they were stored in liquid nitrogen. For the experiments, tumour cells were amplified until passage 26 in RPMI 1640 culture medium (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) supplemented with penicillin (100 UI/ml)/streptomycin (100 µg/ml)/amphotericin B (0.25 µg/ml) (Calbiochem, Burlington, Massachusetts, USA) and fetal calf serum (FCS, 10%) (Pan Biotech, Aidenbach Bavaria, Germany).

The cultures were grown in a plastic cell culture flask at 37ºC and 5% CO_2 in a humidified incubator (MCO-19AIC UV, Sanyo, Moriguchi, Osaka, Japan). When subconfluence was reached, cells were detached from the culture flask using Trypsin-EDTA (Invitrogen, Waltham, Massachusetts, USA). Subsequently, they were suspended in culture media supplemented with FCS (20%) and centrifuged at 1500 rpm for 5 minutes in a refrigerated centrifuge, and then the pellet was resuspended in Hank's solution (Invitrogen, Waltham, Massachusetts, USA).

For this study, we used 3-month-old male WAG/RijHsd rats, weighing 250-280 g. Animals were housed in a temperature- and humidity-controlled room with a 12 h light/dark cycle and free access to water and a standard laboratory diet.

To induce liver metastases, about 25,000 CC531 syngeneic cells suspended in 0.05 mL of Hank´s solution were injected under US guidance into the left lateral liver lobe. Fifty rats received tumour cell injection, as described. Twenty days later, any animals not showing tumour development on US were sacrificed. Of the thirty-eight animals bearing liver tumours, ten were randomly assigned to this study, the others being allocated to other experiments. Another ten animals of the same age and sex were used as controls.

Ultrasound imaging

All US examinations were carried out using a MyLab 60 Xvision system (Esaote, Genoa, Liguria, Italy), equipped with a multifrequency linear probe operated at 18 MHz and the focus was set at a depth of between 0.5 and 1.5 cm. Before US, hair of the abdominal region was shaved using a trimmer and the region was denuded using commercially available hair removal cream (Veet, Reckitt Benckifer, Granollers, Spain). During US sessions, rats were kept under inhalational anaesthesia with isoflurane 1.5%.

On day 0 of the experiment, tumour cells were injected into the rat's liver by US-guided injection, through the midline of the abdominal wall using a 27G needle. Once the tip of the needle was seen inside the liver parenchyma (in the centre of the distal part of the left lobe), tumour cells were slowly infused (Fig 1). Twenty days later, US examinations were performed to assess tumour development. Any animals not bearing tumours were immediately sacrificed.

On day 28, US was performed again to measure the tumour volume. The three largest diameters (cranial-caudate, anterior-posterior and right-left) were measured by US in the transverse and sagittal orientations (Fig 2). Then, using the US system software, tumour volume was calculated from these diameters.

Biochemical and histopathologic analysis

After assessing tumour volume by US, a middle laparotomy was performed in all of the rats to explant the livers of those bearing tumours; blood samples were also obtained and the animals were then sacrificed. In all cases, 5-ml samples of blood were obtained by puncture of the inferior vena cava using a 21G needle. After centrifugation, serum samples were frozen at -25ºC until they were processed.

The blood samples were analysed, in a Cobas® 8000 modular analyser, equipped with Cobas c 702 module (Hoffmann-La Roche, Basel, Basel-Stadt, Switzerland), to measure levels of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (AP), amylase, creatine kinase (CK) and creatinine (Cr) (all chemical reagents for this analysis were purchased from Roche Diagnostics GMBH, Rotkreuz, Zug, Switzerland).

Tumours were explanted, split in half and immersed in paraformaldehyde (4%), dehydrated and finally embedded in paraffin. Then, 3-µm histological sections were taken every 200 µm and stained with haematoxylin-eosin (H/E) to assess tumour necrosis under a microscope (Olympus CH2, Shinjuku, Tokyo, Japan).

Five sections from each animal were observed under a microscope by a skilled pathologist and images were taken of these sections. Then, using ImageJ software® (National Institutes of Health, Bethesda, Maryland, USA), the total tumour area (TTA) and the necrotic area (NA) were measured and, for each of the tumour sections, the NA/TTA ratio was calculated (Fig 3). The NA/TTA ratio allows us to calculate the percentage of the tumour that is necrotic for each tumour sample. The mean percentage of necrosis for each tumour was obtained by calculating the mean of the ratios obtained for the five tumour sections studied from each tumour sample and multiplying this value by 100.

Statistical analysis

Biochemical data, tumour volume measured by US and percentage of tumour necrosis were presented as mean ± standard deviation, as the data were normally distributed. Comparisons between groups were performed using Student's t test. Linear regression analysis was performed to assess the association between

volume measured by US and percentage of necrosis observed in tumour tissue samples. Prism (version v.6, GraphPad Software Inc., California, USA) was used for both types of analysis.

Results

US-guided tumour induction

US-guided cell inoculation was successfully performed and well tolerated in all the rats, without any adverse effects (death, bleeding from the puncture site or weight loss). Analgesia was not required in the following days. The procedures took around 10 minutes for each animal, and the animals fully recovered in less than 5 minutes. A single well-defined tumour implant developed in the left liver lobe in 76% of cases (38 rats), while no tumour growth was found in the others (12 rats). No extrahepatic tumours were observed in our model.

Tumour volume assessment

Thirty-eight single metastases developed, one in each animal. Metastases appeared as well-defined intrahepatic hyperechogenic lesions bulging out of the liver and located inside the left liver lobe (Fig 4). The mean tumour volume assessed by US was 0.61 ± 0.39 ml.

Biochemical and histopathological analysis

Biochemical serum levels in control- and tumour-bearing rats are presented in Fig 5. There were no significant differences between groups in ALT or Cr levels (Fig 5 A and F). In contrast, AST and AP levels differed strongly between the groups ($p<0.0058$ and <0.0001, respectively). Specifically, the AST level was 1.68-fold higher in the tumour group (94 ± 22 vs 57 ± 2.9 $U \cdot ml^{-1}$ in controls; Fig 5 B), while the AP level was 1.3-fold lower (119 ± 8.7 vs 157 ± 14 $U \cdot ml^{-1}$ in controls; Fig 5 C). There were also significant differences ($p <0.05$) in the serum levels of CK (1.19-

fold higher in the tumour group; Fig 5 D) and amylase (1.13-fold lower in the tumour group; Fig 5 E).

Under microscopic observation, healthy liver tissue had a normal structure, typical of this organ. Pathological analyses confirmed the cancerous nature of the growths identified by US: an intestinal-type carcinoma, composed of tumour nodules or clusters of tumour cells, which were moderately or well-differentiated and composed of column-epithelial and caliciform cells (Paneth and neuroendocrine cells) encasing atypical glands. These glands had a mucoid and eosinophilic cytoplasm with epithelial necrotic fragments. The tumours were surrounded by peripheral stroma or capsule-like structures, which clearly mark the interface between tumour tissue and healthy liver parenchyma. This allowed us to delimit the area of the tumour in each sample (88.5 ± 40.1 mm^2). Coagulative necrosis, shadow cells, shrunken and pyknotic nuclei (condensed and hyperchromatic nuclei), and more intensely eosinophilic cytoplasm were also observed within the tumour tissue. These characteristics were used to define necrotic tumour tissue and allowed us to estimate the area of necrotic tissue in each tumour (7.6 ± 3.01 mm^2).

Finally, the percentage of tumour necrosis in each sample was calculated (as described above) from necrotic area and total tumour area, as measured by ImageJ analysis. The mean percentage of necrosis across all the tumours was 8.6 ± 7.7%.

Correlation analysis

US-measured tumour volumes and corresponding necrosis percentages were used to perform a linear regression analysis, and the results were plotted (Fig 6). The linear regression equation was obtained (tumour necrosis percentage = 21 x

US-tumour volume [in ml] - 3.1), as were the coefficient of determination, r^2=0.83, and Pearson's correlation coefficient, r=0.911.

Discussion

In accordance with current legislation on experimental laboratory animals and applying the 3Rs principle, methods need to be developed which minimise the number of animals used in research (de Boo and Hendriksen 2005), besides refinement of the procedures and complete replacement of animals with other approaches. When the goal is to obtain data over the course of a process (e.g., disease progression or treatment), using ultrasound or other imaging techniques, it is possible to perform longitudinal studies in small groups of animals, instead of repeating series to sacrifice animals at different intervals. That is, without imaging, it would be necessary to use much larger series to obtain reliable outcomes from experiments (Seo et al. 2018).

Further, in our field, each experiment requires a control group, which implies assigning half of the induced tumours to the treatment of interest, while the other half receive no treatment. If a tumour model were to be well standardised, a control group might not be necessary and the number of animals in each group could be significantly reduced.

In addition to ethical issues, there are many other reasons for reducing the number of animals used, such as the high costs and difficulty of obtaining some types of animals, and limited space and/or funds for their housing. Both ethical and economic concerns are minimised when invasive or end-point analyses are avoided.

Concerning experiments involving animals, an image-based technique to induce

268 or monitor tumour growth needs to be able to detect small tumour implants
269 (sensitivity), acquire images as fast as possible (rapidity) and produce high-
270 resolution images (quality). In addition, it should be relatively easy to operate, not
271 too expensive to purchase and/or maintain, non-invasive and as safe as possible
272 for both the operator and the animal. US has almost all of these characteristics: it
273 is a nonionizing technique, no surgical procedure is necessary (anaesthesia not
274 being mandatory for the examination) and images could be acquired and analysed
275 at the same time (Cootney 2001).
276 US equipment is comparatively inexpensive, and does not require specially
277 adapted facilities, or the injection of (radioactive) tracers, which is an advantage
278 over other imaging modalities such as magnetic resonance imaging, computed
279 tomography (CT) or micro-CT, or positron emission tomography. Nowadays, small
280 animal imaging platforms based on CT are also available and do not require
281 bunkers, but they are still so expensive that they are beyond the means of many
282 research groups.
283 On the other hand, it is also true that US has some limitations. In spite of not
284 being necessary to hold an operator's certificate, a high level of expertise is needed
285 to avoid over- or underestimation when tumours are being measured. Secondly, it
286 is extremely important to consider which body structures or tissues the US is
287 travelling through, as, in particular, US signals are unable to penetrate far into bone
288 or gas-filled structures (Cootney 2001). This is not a major issue in our model
289 because of the tumour implantation site. The injection site (distal part of the left lobe
290 of the liver) is not affected by shadowing from bones (such as ribs) and is superficial
291 to abdominal organs containing gases (i.e., the stomach and bowel). Small image
292 resolution is another important issue in US studies. Resolution is directly related to

US frequency, but increasing US frequency reduces imaging depth. However, this potential limitation does not apply to the use of US in rodents because using high frequency probes (18 MHz clinical probes being used in our study) high resolution images can be achieved while reaching the tumours to study (10-15 mm depth). Another mayor limitation of US-imaging is signal-to-noise ratio (SNR); however, as the SNR increases as the distance between the probe and the organ is reduced because we work with small animals, the distance is so short that the SNR is high enough, not affecting the resolution (Balaban and Hampshire 2001).

It is true that rat models have not been widely used when studying new treatments for cancer; in fact, most studies have used mice as the experimental animal model. Nonetheless, sometimes it is not technically possible to use them because of their size. When new therapies based on systemic administration (oral or intravenous) are under study, their small size is not a major issue, but when, for example, selective delivery using an intravascular approach is being investigated, the mouse is not the preferred animal model. In this study, we chose a rat experimental model because we are interested in site-specific delivery of treatments through intravascular administration (Echevarria-Uraga et al. 2012a; Echevarria-Uraga et al. 2012b).

The tumour induction mechanism is one of the most debated topics in cancer research involving the use of laboratory animals. Direct injection of tumour cells into the site selected has been widely described in the literature, though it does not involve the migration of malignant tumour cells to the growth site. Previously reported studies (inducing tumour implants by direct injection) have achieved well-defined tumour implants in 60 to 70% of the animals injected (Adwan et al. 2017; Arriortua et al. 2016; Echevarria-Uraga et al. 2010; Echevarria-Uraga et al. 2012a;

318 Kim et al. 2009; Robertson et al. 2008). This rate is quite close to that previously
319 reported by Chan in 2010 working with a hepatocellular cancer rat model (Chan et
320 al. 2010) and similar to that achieved in our study, in both cases using US-guided
321 injection, but 20-30% lower than that obtained in the intraportal administration
322 model described by Thalheimer (Thalheimer et al. 2009). For this study, we opted
323 for the injection technique because it reduces variability in the number, size and
324 growth rate of tumour implants.

325 Focusing on total procedure time, the US-guided procedure takes twice as long
326 as direct liver injection, as reported by our working group (10 vs. 4-5 minutes,
327 respectively) (Herrero de la Parte et al. 2015). Though other authors, such as
328 McVeigh et al. (McVeigh et al. 2019), working on BALB/c nude mice, have reported
329 US-based procedure times very similar to those for direct injection (5 minutes), it is
330 important to take into account the differences between the procedures and the
331 animal models used. Here in, we describe the technique for a non-nude animal
332 larger than that used by McVeigh et al. which means a large volume of cell
333 suspension (500 µl vs. 40 µl, respectively) and the need to completely remove
334 abdominal hair (which takes around 2 to 3 minutes). Moreover, mice induction takes
335 around 15 to 30 seconds (Szczęsny et al. 2004), whereas in rats, this procedure
336 takes considerably longer, as long as 222 ± 42 seconds (Wren-Dail et al. 2017).

337 Further, US-guided injection is associated with a shorter total recovery time and
338 less post-procedure pain, since it is not necessary to perform a laparotomy to
339 expose the liver for tumour cell injection and post-procedure analgesia is not
340 generally required. Notably, in this study, US-guided induction obtained a similar
341 success rate to that in conventional surgically-based models, but it seems to be
342 less harmful and better tolerated by the laboratory animals.

The development of areas with low perfusion and necrosis is a natural process associated with the natural growth and spreading of tumours. Therefore, characterizing and improving our understanding of this phenomenon is key in all experiments involving animal models of tumours. Further, the appearance of necrosis must be considered when analysing results of a new treatment, and it should not be attributed to a therapeutic effect alone. In our experimental model of colorectal cancer liver implants, histological analysis of tumour sections shows that the mean tumour necrosis percentage attributable to natural phenomena is 8.6 ± 7.7%, though may be as high as 35% in larger tumours. Another study, conducted by our group analysed tumour necrosis percentage after focused hyperthermia therapy, and found a median tumour necrosis percentage of 20%, with a very wide range of tissue necrosis (3–99%) (Arriortua et al. 2016).

Assessing the association of this necrosis with tumour volume estimated by US, the Pearson's correlation coefficient, according to Colton (Colton 1974), shows a strong correlation between necrosis percentage and tumour volume. From this, we can conclude that our study presents a novel and relatively inexpensive method for exploring how a given treatment might induce necrosis of hepatic tumour implants without requiring untreated animals (control group). This correlation has only been demonstrated for this tumour model, however, and we do not know whether a similar correlation would be found in other tumour models.

Serum chemistry parameters are also an important factor to take into account when testing new experimental treatments. These parameters allow us to assess whether the treatment or the disease itself affect the physiological status of animals. In our model, serum hepatic, renal, pancreatic and general enzyme levels were analysed in both control and tumour-bearing animals. Though we observed

significant differences between enzyme levels in tumour-bearing and tumour-free rats, in all cases, the values lay within the range that can be considered normal, as provided by the animal supplier and documented in the literature (Bolant Hernandez et al. 1989; Bolant Hernandez et al. 1990; McGovern et al. 2015). It can therefore be concluded that tumour development seems not to have a significant impact on the general, hepatic, or renal status of the animals.

Conclusions

US-guided cell injection does not differ from direct injection in terms of tumour-development rate. On the other hand, it is a much more time-consuming procedure for the researcher, but shortens the recovery time for the animals.

Our correlation analysis shows a strong association between tumour necrosis percentage and tumour volume, which means that the US-measured tumour volume can be used to estimate tumour necrosis percentage attributable to the volume of the tumour implants.

Tumour development, despite inducing some significant changes in serum enzyme levels, does not seem to affect the animals' general status, levels remaining within normal limits.

In conclusion, our study has revealed that US in our animal model for liver metastases allows us to induce and monitor tumour development and to assess the natural necrosis developing in the tumour.

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