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EDITORIAL

The hepatic sinusoidal endothelial lining and colorectal liver metastases

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Abstract

Colorectal cancer (CRC) is a common malignant disease and the severe nature of cases in men and women who develop colorectal cancer makes this an important socio-economic health issue. Major challenges such as understanding and modeling colorectal cancer pathways rely on our understanding of simple models such as outlined in this paper. We discuss that the development of novel standardized approaches of multidimensional (correlative) biomolecular microscopy methods facilitates the collection of (sub) cellular tissue information in the early onset of colorectal liver metastasis and that this approach will be crucial in designing new effective strategies for CRC treatment. The application of X-ray micro-computed tomography and its potential in correlative imaging of the liver vasculature will be discussed.

Key words: Apoptosis; Australia; Correlative microscopy; Endothelial cells; Hepatic metastasis; Colorectal cancer; CC531; Gaps; Interferon gamma; Kupffer cells; Natural killer cells; Nitric oxide; Macrophages; Modeling; Phagocytosis; Plugging; Pit cells; Stellate cells; X-ray micro-computed tomography

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INTRODUCTION

Colorectal cancer (CRC) is a common malignant disease, with the majority of deaths attributable to hepatic metastasis. In the Western world, it is the second cause of cancer death in women after breast cancer, and the third cause of cancer death in men after lung and prostate cancer, being responsible for about 492 000 deaths p.a. worldwide and 4500 deaths p.a. in Australia[1]. In addition, the statistical data reveal that the risk of developing CRC disproportionately strikes individuals in the age group 65 years and older, illustrating its health longevity impact on the ageing population. At the first diagnosis of CRC, 20% of the patients already have liver metastasis and 30% of the patients will develop metastasis afterwards. 80% of the patients who die of CRC have metastases in the liver and prognosis is generally poor[2].

It is obvious that once the tumour cells have immigrated to the liver, they cross the hepatic sinusoidal endothelial barrier and by the time liver metastases form, most steps in the metastatic cascade have been completed. Consequently, exploring the preceding stages of CRC metastasis, proliferation and new blood vessel formation as well as mechanisms to disturb cell survival are to date of main interest as they are largely unexplored. As discussed in the following sections, the availability of new reconstructing and modeling techniques provide liver cancer biologists with an invaluable tool to bridge the gap between bench science and the development of potential novel liver CRC (immuno) therapeutic strategies.

COLORECTAL CANCER AND THE HEPATIC SINUSOIDAL IMMUNE SYSTEM

When the tumour cells invade the vascular bed and metastasise to the liver, they encounter the liver specific immune defence mechanisms. This hepatic sinusoidal immune system involves the hepatic-specific natural killer cells (NK) (pit cells)[3], Kupffer cells (KC) (liver-associated macrophages)[4] and hepatic endothelial cells (HEC)[5], and is proven to play an important role in protecting the liver from invading colon carcinoma cells[6]. The conventional paradigm of CRC liver metastasis is based on a multi-step process characterized by a series of structural, cellular and molecular events, which give the tumour cells the ability to proceed through the many phases of liver metastasis. Based on literature survey the following common sequence of key-events within the liver sinusoids are involved in the

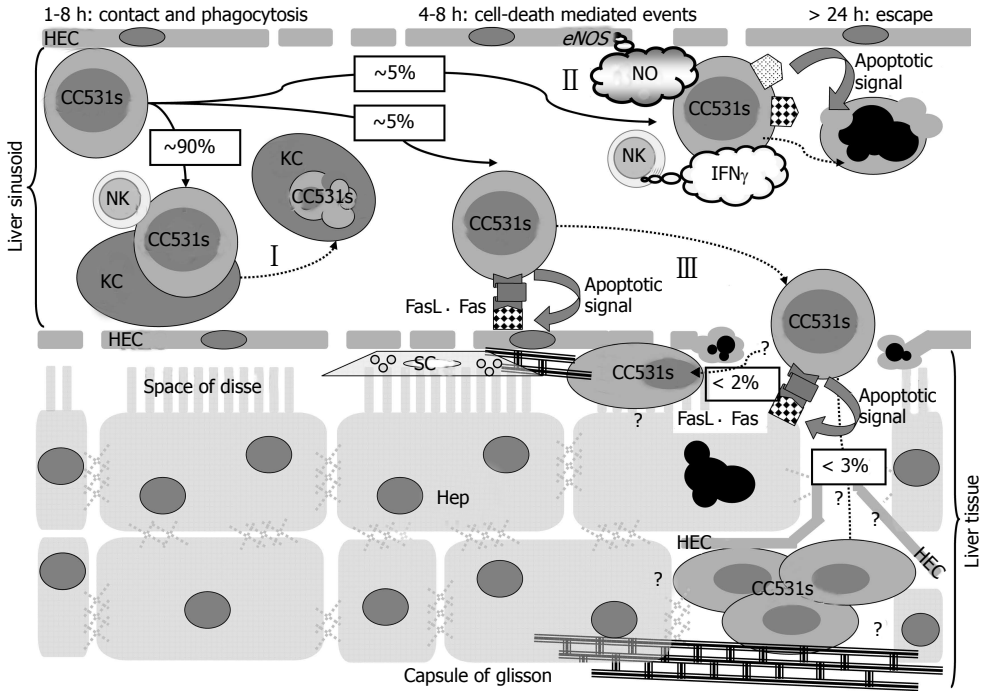

Figure 1 Schematic representation of the local hepatic sinusoidal immune defence system in the different steps of CRC liver metastasis (Steps Ⅰ to Ⅲ) as outlined in detail in the "*Phagocytosis, Apoptosis, Endothelial Retraction and Tumour Survival*"-section. The subsequent steps in the metastatic cascade as denoted by question marks illustrate that considerable work remains, especially once CRC cells traverse the sinusoidal endothelial lining and invade the liver tissue.

process of CRC liver metastasis: Tumour cells approach the liver tissue through finer branches of the portal vein, where they will be trapped in either the finest branch of the portal veins, or the portal hepatic sinusoids. These sinusoids are narrow and tortuous. The diameter of sinusoids is much smaller than the diameter of CRC cells. NK and KC are mobile cells and can be seen moving along the hepatic sinusoid in *in vivo* microscopy experiments (our unpublished data). At this stage, the NK and KC are seen to be associated with the majority of the tumour cells, resulting in cytolysis and subsequent phagocytosis of the majority of the cancer cells[7-9]. Subsequently, specific adhesion of tumour cells within the hepatic microcirculation and active extravasation of the surviving cancer cells through the damaged hepatic endothelium takes place[10]. Both are believed to be essential events for cancer metastasis in the liver[11]; although others postulate that tumour cells develop exclusively intravascularly during the early stages[12]. However, it has been demonstrated that endothelial damage and gap formation occurs in both scenarios, enabling large cellular surface interactions between the tumour cells and the hepatocytes[9-11].

In a later stage, matrix proteins derived from the stellate cells (SC) (liver-associated fibroblasts) are believed to provide a substrate for migration of tumour cells and infiltrating immuno-competent cells, whereas later on tight structures of matrix proteins surrounding tumour nodules provide a barrier for establishment of direct KC- and NK-cell-to-tumour-cell-contact and/or targeted therapies[9,13]. This is supported by the observation that large numbers of KC and NK cells were not activated at later phases in the metastasis process[14]. Finally, CRC cells spread throughout the liver, followed by the abdominal and peritoneal cavity[9,15].

PHAGOCYTOSIS, APOPTOSIS, ENDOTHELIAL RETRACTION AND TUMOUR SURVIVAL

Our previous microscopy and fine structural immunochemistry studies significantly contributed to the above model and as depicted in figure 1 we were able to demonstrate that KC, NK and HECs all work together in concert as one immune-surveillance guardian in the defence against CRC liver metastasis (Figure 1)[6]. Furthermore, it was shown that phagocytosis and apoptosis are key processes in three central steps in the complex CRC liver metastatic cascade, briefly: (Step Ⅰ) When CC531s colon carcinoma cells encounter the liver sinusoid about 90% of the tumour cells are eliminated by a synergistic action between KC and NK cells[9]; (Step Ⅱ) We have proven that HECs express FasL and that about 5% of the colorectal CC531s cell population express functional Fas under influence of IFNγ and NO released in the sinusoid by NK and HECs, respectively. In this situation, the IFNγ-activated pathway supports the immune system by inducing apoptosis in CC531s cells[16]; (Step Ⅲ) Conversely, Fas expressing HEC undergo apoptosis by FasL expressing CC531s cells. As a result, about 5% of the CC531s cells are able to escape the local immune system and provide themselves a gateway towards the liver tissue as the HECs retracts. Next the CC531s cells have free access to the Fas expressing hepatocytes (Hep) which undergo in turn apoptosis by the FasL bearing CC531s cells and as a result invade the liver tissue[17]. Reconstruction data obtained *via* the aid of confocal laser scanning microscopy indicated that surviving cancer cells are primarily confined to the Space of Disse and to the Glisson capsule, suggesting that metastasis would initiate from this extracellular matrix-rich region.

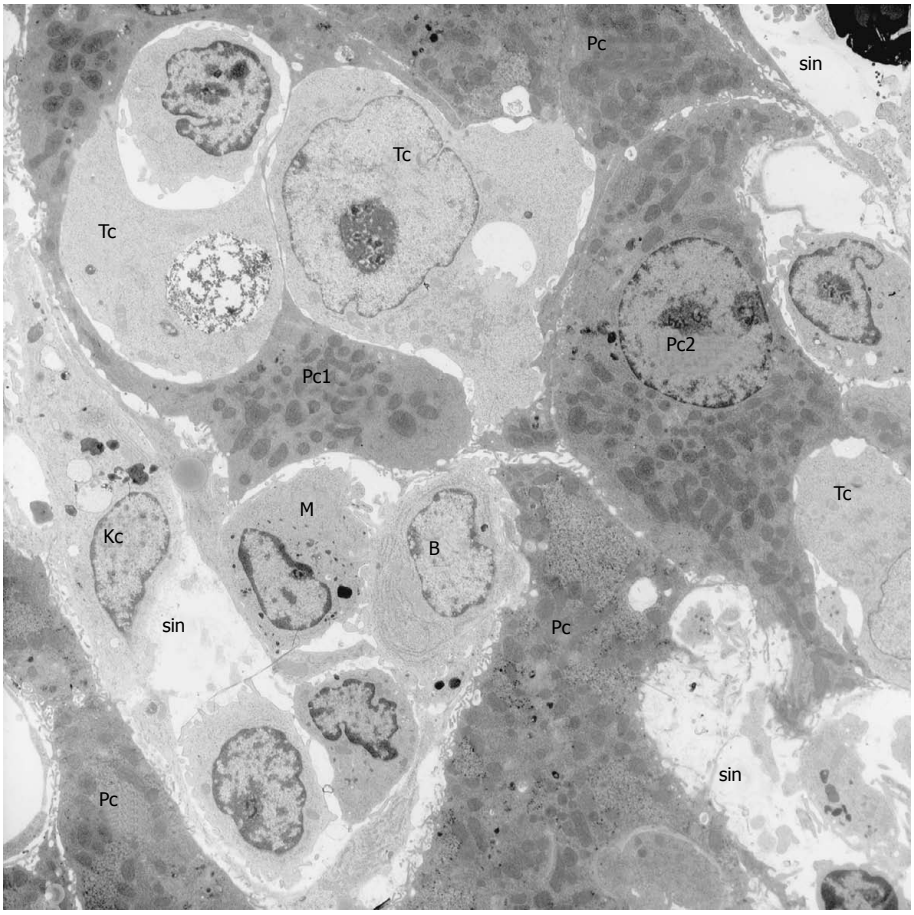

Figure 2 Transmission electron microscopy photomicrograph at low magnification (6700 x) of rat liver one week after i.v. injection of one million CC531s colon cancer cells. Tumour cells (Tc) can be recognized by a ribosome-rich, bulky cytoplasm containing few organelles. Tumour cell nuclei are large and contain mostly euchromatin. In this picture, tumour cells have already taken position in the liver parenchyma and compress or deform the parenchymal cells (Pc1 and Pc2). In this stage, tumour cells are so numerous that defending cells, such as Kupffer cells (Kc) and monocytes (M) in the hepatic sinusoids (sin) and B lymphocytes (B) seem to be no longer involved as in earlier stages. At this stage of tumour cell settlement, rebuilding of the hepatic tissue sets in and soon small visible white spots of metastasis will be seen on the surface of the liver.

Overall, based on our combined light-, laser- and electron microscopic *in vivo* and *in situ* studies, we believe that the majority of tumour cells entering the liver will be destroyed in this first confrontation with hepatic sinusoidal cells. The trouble is supposed to be the fact that only a very few tumour cells need to escape from the hepatic sinusoids and after settling in the liver parenchymal tissue, as depicted in the photograph (Figure 2), they form metastases and start spreading new tumour cells in the hepatic tissue (i.e., secondary metastasis). It is also supposed that after leaving the hepatic sinusoids, the tumour cells are not chased by the local hepatic sinusoidal immune cells, and that the liver tissue has to rely from that moment onwards on other cellular- and immune defence systems, such as those offered by cytotoxic T cells, monocyte-derived macrophages and others.

RECONSTRUCTING AND MODELING COLORECTAL HEPATIC METASTASIS WITH CORRELATIVE THREE-DIMENSIONAL IMAGING TECHNIQUES

In the past, mainly two-dimensional structural tissue information has been generated about the metastasis pathways of CRC tumour formation in the liver. Attempts to target specific CRC pathways for therapeutic intervention, such as apoptosis by cell-death mediated drug delivery and/or new blood vessel outgrowth by specific anti-angiogenic drug release, partly failed because of our limited structural and molecular understanding about the tumour's complex cell- and tissue microenvironment using two-dimensional imaging. New concepts and progress in cell biology have been discovered thanks to improved correlative microscopy techniques that continue to rely predominantly on advances in new, three-dimensional (3D) reconstructing and modeling techniques[18,19]. Ideally, 3D correlative microscopy can be defined as an imaging platform aiming to cross-correlate information with multiple microscopy techniques on the same tissue(s) and/or cell(s). There is growing evidence from the literature that this approach facilitates the understanding of cellular and/or rare events by providing the possibility to collect new qualitative and quantitative information in a large sample volume[20,21]. In the past our group successfully applied different biomolecular microscopy methods on our CC531s CRC model. The data conferred under the above section were obtained by combining confocal laser scanning-[9,22], live cell-[23], atomic force-[24], scanning electron-[10,17] and transmission electron[16,25] microscopy data. In line, we started up correlative confocal laser scanning microscopy-[9,18] and X-ray micro-computed tomography (CT)[26] studies as defined above to quantitatively collect the spatial and temporal cell- and tissue architecture at different resolution levels of CRC hepatic metastasis (*vide infra*).

In a recent study[27], we applied the impregnation method in which en bloc staining of perfused-fixed hepatic tissue with osmium tetroxide and uranyl acetate was shown

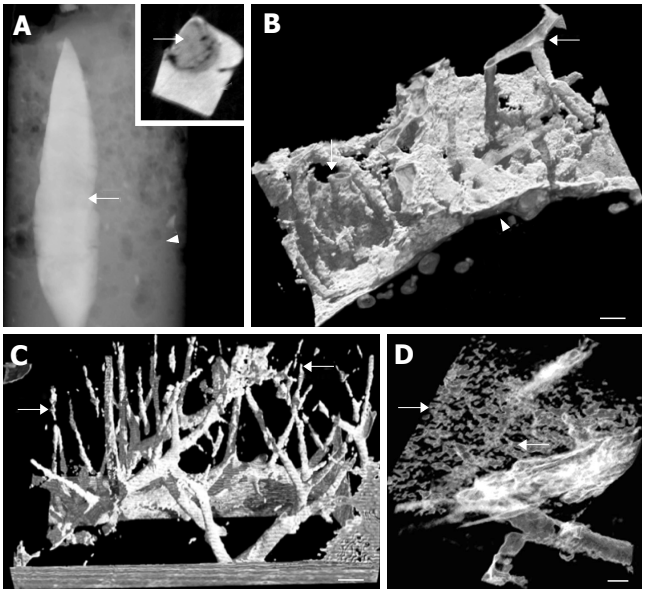

Figure 3 X-ray micro-computed tomography (CT) image set of hepatic tissue. For detailed recording and subsequent image processing settings we refer to Ananda *et al* (27). **A**: Low -magnification data showing an overview of a glutaraldehyde perfused-fixed and subsequently osmium tetroxide/uranyl acetate *en bloc* stained liver lobule (arrow) versus the unstained sample mounting support (arrowhead). Note, inset shows the corresponding Z-info at an ad random height of the main image; **B**: X-ray micro CT 3D reconstruction image showing the portal venous blood vessels (arrow) and hepatic tissue (arrowhead); **C**: X-ray micro CT reconstruction after density histogram image filtering, showing the intricate pattern of hepatic sinusoids (arrow) in 3D context. Note the absence of liver tissue information under this image processing condition (compare with B for the difference); **D**: Conversely, using different opacity thresholds resulted in the visualization of the hepatic cords, i.e. parenchymal tissue, (arrow). (Courtesy to Ms. S. Ananda and Ms. V. Marsden). Scale Bars (B-D), 500 μm.

to be successful to reconstruct and model a large sample volume of the macro- and microvasculature of hepatic tissue with X-ray micro-computed tomography (Figure 3). Furthermore, we demonstrated that correlative laser light optical imaging provided a limit of confidence for X-ray micro CT imaging of the hepatic vasculature as the large blood vessels such as the hepatic portal veins, and the smaller blood vessels i.e., the hepatic sinusoidal vascular bed could be visualized[27]. When applying the established contrasting method to the CC531s CRC metastasis model[9] it was observed that the dense array of blood vessels was interrupted in the liver tissue samples bearing the CRC cells when compared to the controls[27]. This is in line with the earlier observations made by Maehara[28] who showed tumour-induced reorganization of the auricular vascular bed after barium sulphate contrasting. Based on these data we forth casting the ability to correlate information in large tissue volumes from the X-ray micro CT models with reconstruction data obtained from confocal laser scanning microscopy on the same sample and region of interest by using fluorescent- and X-ray dense fiducial markers. This will definitely bridge the resolution gap from the micrometre to nanometre scale in studying cancer-mediated events, respectively.

In conclusion. Advanced multidimensional correlative microscopy methods and modeling techniques will feature heavily in the future quest to understand and to quantitatively define the spatial and temporal mechanisms regulating the pathways of CRC liver metastasis. There will be no doubt that elucidating the subcellular and molecular events of tumour-mediated cell-death processes (apoptosis) and collecting simultaneously additional structural and functional data in tumour-induced new blood vessel formation (angiogenesis) will be of main interest in future combined imaging studies as this approach will most probably result in an accurate correlative representation of the liver architecture and the tumour's microvasculature. One may hope that the combination of correlative insights gathered will be a breakthrough in the study of liver tumours and this will facilitate novel targeted cancer therapies in the long-term.

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COMMENTS

Background

Major challenges such as understanding and modeling colorectal liver metastases pathways rely on the understanding of simple models derived from large biomolecular image data sets.

Research frontiers

This paper elegantly demonstrated that the development of novel standardized approaches of multidimensional (correlative) biomolecular microscopy methods facilitated the collection of (sub) cellular tissue information in the early onset of colorectal liver metastasis and that this approach is crucial in designing new effective strategies for colorectal cancer treatment.

Innovations and breakthroughs

Advanced multidimensional correlative microscopy methods and modeling techniques will feature heavily in the future quest to understand and to quantitatively define the spatial and temporal mechanisms regulating the pathways of colorectal cancer liver metastasis.

Applications

There is no doubt that the "correlative insights"-approach, as discussed in depth in this "invitation expert review"-paper, will be a breakthrough in the study of liver tumours and will facilitate the design of novel targeted cancer therapies.

Terminology

Colorectal cancer is a common malignancy that frequently metastasizes to the liver. Identifying the mechanisms regulating the nanobiology of colorectal liver metastasis, as well as gaining a better understanding of the interaction between the metastatic tumour cell and the different types of liver cells, including the liver

vasculature, is a first when new therapeutic approaches wanted to be designed.

Peer review

How and why colorectal cancer cells metastasize to the liver has always been a subject of continuing interest. The authors describe this concept nicely, supported by excellent pictures.

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