Avoiding hepatic metastasis naturally: Lessons from the cotton top tamarin (\textit{Saguinus oedipus})

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Abstract

Much has been written about hepatic metastasis and animal models abound. In terms of the human experience, progress in treating this final common pathway, a terminal event of many human malignancies has been relatively slow. The current thinking is that primary prevention is best served by early detection of cancer and eradication of early stage cancers by screening. Some cancers spread early in their course and the role of screening may be limited. Until relatively recently there has not been a pathfinder model that makes the evasion of this unfortunate event a reality. This review discusses such an animal model and attempts to relate it to human disease in terms of intervention. Concrete proposals are also offered on how scientists may be able to intervene to prevent this deadly progression of the cancer process.

Key words: Cotton top tamarin; Hepatic metastasis; Carcinoembryonic antigen; Fibulin-5; Common marmoset

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Core tip: Hepatic metastasis is a terminal event. Avoiding this complication would prolong life and current understanding of inflammatory mediators allows possible secondary intervention. The cotton top tamarin (CTT), like humans develops inflammatory bowel disease complicated by colorectal cancer but avoids liver metastasis. We suggest 5 mechanisms by which CTT avoid liver spread. They involve changes in ICAMs and their receptors, carcinoembryonic antigen (CEA) family mediators of angiogenesis, post translational modifications of molecules like CEA, and increased expression of anti-proliferative agents such as fibulins. This changes our perception from “monkey
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see, monkey do” to “see what the monkeys do and do the same”. Possible avenues of intervention are suggested.

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INTRODUCTION

There are many animal models for human cancer and most of these do not occur naturally but are designed to mimic human disease by introduction of mutagen and may also employ promoters. Natural models lack this human "design" element but historically the differences may enhance understanding of disease causality and management for example, the Roux Sarcoma virus in the Plymouth Barred Rock fowl (chickens) and Bittner milk factor in murine mammary carcinoma among others. It is however unusual for a single animal to provide more than 1 natural model for disease.

Enter the cotton top tamarin (CTT), a pint-sized lower order, New World primate of the order Callitrichidae. This remarkable monkey provides a model for human disease in inflammatory bowel disease and potential mechanisms, cancer of the lymphatic system, large bowel cancer, and immune-altered states, and recently liver metastases. While a review of these models apart from the latter is beyond the scope of this review, it is significant that the example of the liver metastasis model could best be described as a "negative" or "avoidance model" in that the CTT with colon cancer avoids liver metastasis with an estimated frequency of 97.2%.

The hypotheses of how the CTT achieves this are open for discussion and debate has been ongoing since the first symposium, reported in 1985 where anatomical blood vessel variance was advanced as an explanation. This was refuted by veterinarians since the anatomy is similar to most mammals including humans (N. Clapp personal communication). Our group has been exploring hypotheses based on adhesion molecules known to be important in human liver metastasis. While the article will focus on these molecular families in tamarins, it is important to first discuss our understanding of their importance in human "metastogenesis" as the findings in the CTT may enable intervention in the human where over 70% of GI cancer victims succumb to death caused by hepatic metastases.

Hypotheses of hepatic metastases in humans

Cancers of the colon and rectum comprise 14.5% of all cancers diagnosed in the United States (149880) and 11.1% of cancer deaths (51710) each year. The primary treatment for large bowel cancer is surgery. Patients who recur do so largely in the liver, lung or peritoneum. While combination chemotherapy can increase survival even in advanced disease it is rarely long term. Metastatic colorectal cancer is therefore a major public health problem. Colorectal cancer is associated with chronic inflammation. Inflammatory cytokines can be induced by interaction of terminally differentiated macrophages with the cancer associated glycoprotein carcinoembryonic antigen (CEA). CEA (CEACAM5) is a large heavily glycosylated glycoprotein of indeterminate function in the normal tissue in which it is expressed. Discovered in 1965 by Gold and Freedman it has become the most used tumor marker for colorectal cancers but failed to live up to the early expectations for use as an early detector of cancer. It is a glycoprotein that is a member of a large gene family that consists of 29 genes divided into three subgroups that include the CEA-like glycoproteins and the pregnancy-specific glycoproteins (PSGs). These CEA like proteins are members of the much larger immunoglobulin supergene family. The nomenclature for the entire CEA and PSG families is published in Beauchemin et al.

CEA

CEA is a GPI anchored glycoprotein with an average MW of 180 kDa this varies depending on the degree of glycosylation of the native protein. CEA contains 651 amino acids and these are found in 7 immunoglobulin like domains, an IgV domain at the N-terminus and six disulfide bridge linked IgC domains. There are 29 possible sites for N-linked glycosylation and they tend to be of the complex tetra antennary type. Recently a large number of functions in cancer cells have been attributed to CEA. These include a role in cell-cell adhesion, apoptosis (anoikis), inflammatory responses leading to increased hepatic and possibly lung metastasis and angiogenesis.

CEA AND ITS IMPACT ON LIVER METASTASIS: INDUCTION OF INFLAMMATORY RESPONSES IN THE LIVER

Liver metastasis is the major cause of death in patients with colorectal cancers. There is good evidence that cytokines play a major role in preparing the liver for implantation and subsequent growth of cancer cells. It appears that a relationship exists between the colon cancer derived glycoprotein CEA and macrophage related cytokine production particularly associated with CEA Kupffer cell interactions in metastasis of colorectal
cancers to the liver. The discovery of CEA in 1965[17] marked a turning point in the study of cancer and led what might be called the age of tumor markers. CEA was the first commercially available tumor marker and may be considered the prototype[25]. Serum elevations of CEA (above 3 ng/mL) are seen in about 60% of colorectal cancer patients at presentation[26] and elevated levels are also seen in a number of other cancers including breast, gastric, lung and pancreas. Unfortunately, the early premise that CEA levels could be used as a screening test for colorectal cancer was not fulfilled. CEA was found not to be cancer specific and high false positive and false negative rates precluded its use in early detection of cancer. However, CEA is a useful marker in the determination of prognosis[27] and for detection of recurrence in the post-surgical follow up of colorectal cancer patients[28]. CEA has also been used for the detection of occult tumor by radioimmuno-detection[29] and as a target for treatment using radioimmuno-therapy[30]. In addition a number of studies have used CEA as the target antigen for immunotherapy[31]. Clinically CEA is very well understood, but its biological role is not clear and its function in the normal colonocyte still evades us. However over the years a number of functions mostly associated with tumor cell behavior have been attributed to CEA. We focused on the molecular interactions between CEA and its receptor CEAR in colorectal cancer cells and how disruption of these interactions and inhibition of cytokine production will affect the spread and growth of colorectal cancers. Designing an effective mimetic-based therapy requires the identification of the responsible molecules, mechanisms of action and regulation.

The most important functions of CEA are the effects associated with tumor cell survival whether it is at the primary site or at a distant metastasis. High levels of CEA are also associated with malignant ascites from colorectal cancer (CRC)[32]. CEA interacts with liver Kupffer cells through a cell surface receptor (CEAR) that we have identified as the heterogeneous RNA binding protein (hnRNP) M4[33]. The hnRNP group of proteins are multifunctional and are involved in many cellular events including alternative splicing, translational regulation and packaging of transcripts[34]. These proteins have also been implicated in the regulation of mRNA stability and translation in many cancers[35]. CEAR is a highly conserved protein that can bind both RNA and DNA and can transport mature RNA to the cytoplasm as well as acting as a splicing factor[36]. It can also be expressed both on the cell surface, in the cytoplasm and in the nucleus where it is most commonly found[37]. There are 4 isoforms of hnRNP M known[38]. Two of which we have shown bind CEA[39]. One form (hnRNP M4) which we originally identified as the CEA receptor has a 38 amino acid deletion between the first and second RNA binding domains. The longer form also binds CEA[33]. The two other forms have not been identified other than as proteins on 2D gels. CEAR will also bind to CEA in HT-29 colon cancer cells although the functional significance of this is not known. It has been suggested that it may be involved with the resistance afforded to anoikis[39,40]. Binding to CEAR occurs via a penta-peptide motif (PELPK) located at the hinge region between the CEA’s N-terminal and first immunoglobulin loop domain. This interaction produces cytokines by activating a signaling cascade and these cytokines alter the liver microenvironment such that it becomes more hospitable to the implantation and growth of the cancer cells[41,42]. Production of both IL-6 and IL-10 by CEA stimulated Kupffer cells improves the survival of highly metastatic human colorectal cancer cells in the nude mouse intra-splenic injection model for liver metastasis[43] (see Figure 1).

CEA AS AN ADHESION MOLECULE

In 1989 Benchimol et al[42] demonstrated the first potential function for CEA. By transfecting tumor cells with the CEA gene they showed increased cell to cell adhesion and demonstrated that this could be inhibited using anti CEA antibodies. Adhesion was due to interactions between the N-terminal and the 5th and 6th (A3B3) immunoglobulin domains[44]. This suggested that these interactions may play a role in tumor cell behavior and in the development of metastases.

CEA AND ANOIKIS

Another important function that has been attributed to CEA expression is inhibition of apoptosis in particular the apoptosis caused by absence of attachment of cells to a substratum (anoikis)[23,40]. This would play an important role in metastases formation to a distant organ as unattached tumor cells in the circulation would be more susceptible to anoikis.

CEA AND ANGIOGENESIS

Here, we suggest a relationship between CEA, its receptor CEAR and angiogenesis. Others have also suggested a relationship between CEA and angiogenesis though they did not establish it as causal or identify potential mechanisms[45,46]. Recently, however they showed that secreted (soluble) CEA can directly activate endothelial cells via integrin β3 signaling[24,47]. We suggest that an alternate mechanism may also exist involving the relationship between CEA and its receptor CEAR. Interaction of CEAR with CEA in tumor associated stromal cells particularly macrophages is important. Disrupting that relationship may affect tumor growth and progression.

It is significant that Low-Marchelli et al[48] have shown that CCL2 recruits macrophages to promote angiogenesis. We therefore suggest that colon cancer cells recruit macrophages by secreting MCP-1 and these macrophages are activated by tumor derived
responses in PMA activated THP-1 macrophages. These types of study are important for the development of rational therapies that will enhance those currently available.

OTHER POTENTIALLY PRO/ANTI-METASTATIC MOLECULES STUDIED

**Fibulin-5**
During the initial development of metastasis, the adhesion of tumor cells is mainly mediated through binding of extracellular matrix components to cell surface receptors\(^5\). Interestingly, one study\(^6\) found an association of fibulin-5, the newest member of the fibulin family of extracellular matrix glycoproteins, with hepatic metastasis of colorectal carcinoma. Since fibulin-5 plays an important role in antagonizing angiogenesis\(^5\-^7\), we sought to confirm the expression of fibulin-5 expression level in the CTT.

TARGETING CEA/CEAR INTERACTIONS AS A MEANS OF INHIBITING CYTOKINE PRODUCTION AS AN ANTI-METASTATIC THERAPY IN COLORECTAL CANCER
Because transformed cells home into tissues from the circulation in a highly selective way through complex molecular mechanisms, it provides a template for targeted therapy. Designing an effective mimetic-based therapy requires the identification of the responsible molecules and their mechanisms of action along with their regulation. The investigation of methods to block or modulate CEAR function *in vitro* and *in vivo* and relating this to tumor growth and development will increase our understanding of the molecular and biological mechanisms involved in the progression of gastrointestinal cancers. The investigation of methods to block or modulate CEAR function *in vitro* and *in vivo* and relating this to tumor growth and development has increased our understanding of the molecular and biological mechanisms involved in the progression of colorectal cancers. We have shown that the binding peptide YPELPK will induce cytokine responses in PMA activated THP-1 macrophages. These types of study are important for the development of rational therapies that will enhance those currently available.

**OTHER POTENTIALLY PRO/ANTI-METASTATIC MOLECULES STUDIED**

CEA

CEA to secrete IL-6 and IL-8 both known pro-angiogenic factors\(^4\). Anti angiogenic factors are also produced (IL-10) and an imbalance between these two competing factions will tend towards pro-angiogenesis. Avoidance hypotheses in the CTT

Our understanding of cancer and metastases in primates began when colon cancer was described in old world monkeys as early as 1914 and then in the new world in inter alia, Goeldi’s monkey in the early 1960s. The latter, *Callimico goeldii* shares the Colombian habitat with the CTT and is a small black-furred New World lower order primate. At that time tens of thousands of CTT were imported by the pharmaceutical industry utilizing the “tamarin alarm reaction” to test the efficacy of anxiolytics although literature to document
this is difficult to come by utilizing electronic search methods. Problems of CTT husbandry arose when the animals developed the “wasting marmoset syndrome”[54] postulated to be caused by a coronavirus but later effectively countered by improving the nutritional intake. The major challenge facing the CTT is the reduction of its numbers in its shrinking natural habitat in northwestern Colombia and most CTT are today found in zoos. As a result the species was listed as endangered in 1973 by the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES)[55]. Since that time, the numbers of articles on the CTT had increased exponentially. This attests to the interest of the scientific community in the CTT specifically. Despite this interest, primate research in the United States is in decline with the closures of the ORAU (Oak Ridge Associated Universities) facility and the NEPC (New England Primate Center) which housed a significant proportion of CTT. The waning of public support and therefore funding opportunities combined with the expense of operations has largely been the cause of this decline. This is regrettable as it is generally not appreciated that monkey publication numbers are an extensive part of the scientific literature (678000 Medline™-listed publications) attesting to their usefulness and their command of the attention of the scientific community. Now, specifically CTT publications which were formerly about one tenth of non-human primate-themed publications (398 total CTT per National Library of Medicine ticket 28045-54653), are in decline in the example depicted graphically (Figure 2).

In view of the importance of this animal model applicable to many models of human disease, a CTT symposium was convened resulting in the publication of numerous articles in a supplement published in the journal Digestive Diseases and Sciences in 1985[9]. Thereafter a comprehensive book on the CTT was edited by Dr. Neil K. Clapp and published in 1993 by CRC Press[2]. This review seeks to build on that experience specifically focusing on the ability of the CTT to avoid hepatic metastases even though a large proportion of the animals develop advanced colorectal cancer. This is a unique feature of the CTT not typically found in other marmosets. It would appear that CTT peritoneal metastasis and ascites is also not common as it is with terminal human disease.

**CTT MODEL FOR HEPATIC METASTASIS**

The CTT develops colitis in the juvenile years and about one third (34.5%) go on to develop colon cancer and die of the disease. An observational study showed only 1.2% of animals had hepatic metastases[56] (Figure 3).

The etiology of the cancer seems to be the de novo inflammation-dysplasia-cancer variety although animals with adenomas have been described[56]. The risk of cancer deaths shows an increase from 3-8 years and then a gradual decline after age 12 similar to the recently described risk in humans with positive family history of CRC[57]. The etiology is still unknown but stress and micro-organisms may play a role[53,4]. Over half have multiple primaries diffusely distributed but the descending colon has the greatest proportion similar to the rectosigmoid colon predominance in ulcerative colitis cancer location.

**CTT COLON CANCER BIOLOGY VIS A VIS HUMAN**

Despite similarities in cancer biology between CTT and humans it can be seen from Table 1 that the anticipated mutations are either not described or characterization has not been attempted.

When considering changes associated with metastases[63] no CXC, CXCR4, CXCL12 (fusins), CCR7 (chemokine receptor 7) chemokines thought to be important in metastases have been described as yet in the CTT. Interestingly CXCL12 has been described in the common marmoset[64] and thus likely to be expressed in the CTT but is not necessarily a given.
The human kallikrein zymogens (hk2) closely related to prostate specific antigen (PSA) have been studied in the CTT\[65\]. They concluded that the CTT has no functional hk2 or PSA. Since these moieties regulate cancer cell survival and growth the CTT may be a natural knock-out model for the study of this topic. We see in these findings yet another potential mechanism by which the CTT impedes cancer proliferation. Likely Toll-like receptors (TLR) also remain to be discovered in the CTT and may have likely similar functions to the human.

Since lectins\[66\] and adhesion molecules have been described in the CTT we have focused our attention on these molecules, particularly CEA. We also explored extracellular matrix proteins as the interaction between these proteins and cells play an important role in tumorigenesis and metastases. One such protein, fibulin-5\[51-53\] has calcium-binding EGF-like domains and associate with vascular and vascular structures. It is thought therefore to have anti-angiogenic actions and be an inhibitor of metastasis. It has not as yet been studied in the CTT.

**CEA HOMOLOGUES IN THE CTT AND HUMAN**

Cross reactive CEA species in monkeys were known when we published our seminal paper in 1994\[67\]. Haagensen et al\[68\] had reported CEA in higher order non-human primates, including the chimpanzee and gorilla. A mouse analogue had also been described by Abraham Fuks’ group in 1989\[69\]. Our initial experiments used a number of antibodies to CEA including the T84.66 antibody developed by Dr. J. Shively’s laboratory group at the Beckman Institute at the City of Hope which was used in the CEA kit commercialized by Roche™. This antibody when used in immunohistochemistry produced consistently negative results when using colon CTT fixed tissues and later native antigen in CTT colon tissue extracts in early experiments\[70\]. We tested other antigens such as organ specific neoantigen\[71\], thought to be the mediator of the lymphocyte adherence inhibition test for cancer and found reactivity\[72\]. In the early 1990’s Karel Kithier of the Department of Pathology at Wayne State University was beta-testing a CEA kit produced by Tosoh Medics (CEA-AIA PACK kit™) which seemed to have a greater proportion of positive results when compared to another CEA kit marketed by Abbot. These results were expanded and confirmed and found to yield similar levels in CTT and humans with IBD\[60,73\].

In order to show the CEA molecular moieties recognized by the kit we obtained both solid phase and tracer antibodies from Hybritech Inc., from Dr. Harry Rittenhouse. The resultant Western blotting\[67\] showed a specific 50 kDa band shared by CTT and humans (Figure 4).

A control blot using T84.1EC monoclonal which reacts with both NCA and CEA did not recognize the 50 kDa shared antigen in the same samples. Based on the known epitopes on the antibodies concerned we were able to speculate that the shared antigen included the Ig-like loop domains however the exact structure of this moiety remains unknown. In this publication we did hypothesize that “the smaller CTT CEA molecule might lack the critical peptides necessary for uptake and hence explain the paucity of liver involvement in CTTs with CRC”.

**EXPRESSION OF THE CEA GENE FAMILY OF PROTEINS IN THE CTT AND HUMAN**

The next question we needed to answer was if an entire repertoire of CEA-family adhesion molecules were expressed by the CTT and if this subspecies was unique in this respect in the Callitrichidae family. In a later paper in 2000\[73\] we explored these questions by expanding the repertoire of antigens and extending our observations to include a close cousin of the CTT, the common marmoset (Callithrix jaccus - CM) which is

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**Table 1 Comparison of cancer genetics in the cotton top tamarin and human**

| Stage of neoplasia       | Sporadic human CRC          | Human IBD-associated CRC | CTT colitis-CRC            |
|--------------------------|----------------------------|--------------------------|----------------------------|
| Normal-appearing mucosa  | APC initiator               | Inflammation leading to mutations listed below | No cell line mutations in APC exon 4 and 15\[10\] |
| Early adenoma/ indefinite dysplasia | Aneuploidy, methylation, Sialyl Tn | Anueplody, methylation, Sialyl Tn, MSI | 90% diploid; no methylation\[10\] |
| Early promoter           | kRAS, COX-2                | c-src                    | kRAS absent\[10\] |
| Intermediate promoter    | DCC/ DPC4                 | p53                     | APC                        |
| Intermediate adenoma/ low-grade dysplasia | c-src                    | kRAS                     | DCC/DPC4 ND; kRAS absent\[10\] |
| Late promoter            |                            |                          | p53 ND; as above for APC exon 4 and 15\[10\] |

Adapted from Itzkowitz and Harpaz 2004\[61\], and Mattar MC, Lough D, Pishvaian MJ, Charabaty A\[60\]. APC: Adenomatous polyposis coli gene; MSI: Microsatellite instability; DCC: Deleted in colon cancer; kRAS: V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; COX-2: Cyclooxygenase 2 gene; c-src: Rous sarcoma gene that encodes for usual C-terminal inhibitory phosphorylation site (tyrosine-527); DPC4: Deleted in pancreatic carcinoma, locus 4; p53: Protein 53 kilodaltons; ND: Not done.
not endangered but does develop colitis uncomplicated by cancer. Aware that the changes likely to influence CEA uptake were at the N-terminal end of the CEA molecule we used antibodies directed at epitope in that region, including the aforementioned Tosoh Medics™ kit and T84.66 antibody to quantify the CEA in colonic washings.

We found that the concentration of CEA in washings was sevenfold times higher than in washings from humans with IBD. T84.66 antibody was able to demonstrate a faint high MW band in a specimen with a patient with histologically proven rectal cancer and IBD is in lane 9, with human effluent samples in lanes 10 and 11 and human meconium in lane 12. Lane 5 contains the positive human CEA control, and the M, markers (M) are in lane 6. An arrow indicates the M, - 50000 band evident also in human extract (lane 5) likely a deglycosylated moiety. (Published with permission of Elsevier and modified). CEA: Carcinoembryonic antigen.

Figure 4 Specific carcinoembryonic antigen bands shared by cotton top tamarin and humans. Western blot using solid phase anti-CEA monoclonal antibody after electrophoresis of a 12% SDS-polyacrylamide gel run under reducing conditions. Relative mobility (M) is shown on the left, and the type of samples loaded at 10 pg protein/lane are shown above the numbered lanes. Cotton-top tamarin extracts are in lanes 1 to 4 and effluent samples in 7 and 8. An extract from a patient with histologically proven rectal cancer and IBD is in lane 9, with human effluent samples in lanes 10 and 11 and human meconium in lane 12. Lane 5 contains the positive human CEA control, and the M, markers (M) are in lane 6. An arrow indicates the M, - 50000 band evident also in human extract (lane 5) likely a deglycosylated moiety. (Published with permission of Elsevier and modified). CEA: Carcinoembryonic antigen.

DEFINITIVE STUDIES ON THE HOMOLOGY OF THE CEA MOLECULE

A comparative study of the C-terminus of the CEA molecule by the Stanners’ group looked at multiple primate and prosimian species. The membrane linkage in CTT and other monkeys was found to be GPI anchorage rather than transmembrane (TM) seen in mice and rats[77]. This paper showed that the more versatile glycoprophatidyl inositol (GPI) linkage with a greater functionality constellation likely confers a tendency to tumorigenesis. Mutational packages evolved over time, one applicable to most primates including humans, and the second confined to the Cebidae radiation of New World monkeys leading to inhibition of cell differentiation. The rate of Ka (indicator of selective pressure acting on a protein-coding gene) nonsynonymous mutations for this radiation is 7X higher than the average Ka in primates. This led us to postulate that if such mutations occur at the C-terminus, it is quite feasible that similar mutations occur at the N-terminus where they may affect uptake of CEA, inhibiting hepatic metastases. Having
Table 2 Representative somatic sequences from the PELPK region of carcinoembryonic antigen in humans, cotton top tamarin and common marmoset

| HCEA-A1 PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | CTT-WT PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | CM-WT PELPKISSSSSNKPKVPEKDVAFTCEPETQDA |
|--------------------------------------------|------------------------------------------|------------------------------------------|
| M1-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M2-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M3-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA |
| M4-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M5-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M6-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA |
| M8-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M9-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M10-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA |
| M11-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M12-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M13-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA |
| M14-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M15-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M16-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA |
| M17-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M18-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M19-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA |
| M20-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M21-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M22-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA |
| M23-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M24-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M25-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA |
| M26-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M27-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M28-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA |
| M29-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M30-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M31-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA |
| M32-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M33-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M34-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA |
| M35-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M36-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M37-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA |
| M38-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M39-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA | M40-A1 (CTT) PELPKISSSSSNKPKVPEKDVAFTCEPETQDA |

Sequences changes from those of wild type (germ line) are shown in bold. Published with permission of Springer.

also found common CEA-family and fibulin-5 ligand antigens we resolved to also investigate for parallel pathways of metastasis inhibition.

SUMMARY: SPECIFIC METASTASIS-TARGETED STUDIES IN CTT AND HUMANS

Using DNA extracted from the CTT tissues a Hot start PCR was performed and (1) sequences around the hinge region between the N-terminal and the first loop domain of CEA obtained using a Big Dye Terminator™ sequencing kit. We also immunolabeled human and CTT liver tissue using: (2) BGP (CEACAM1) antibody kindly supplied by Christophe Wagener (University of Freiberg, Germany); (3) CEAR (CEA receptor) polyclonal antibody; and (4) an antibody directed to fibulin-5 supplied by W. P. Schiemann (Department of Pharmacology, University of Colorado, Aurora, CO, United States). Finally, (5) we deglycosylated CTT CEA to determine the degree of N-glycosylation, as glycosylation may correlate with hepatic metastases. We thus examined 5 potential ways in which the CTT may evade hepatic metastases in 10 CTT and 25 humans.

To briefly summarize the observed results (Table 2) we enumerate: (1) 63% of the CTT had non-synonymous PELPK pentapeptide mutations shown to be essential for hepatic uptake of numerous groups of protein including CEA and stromelysin, laminin, complement protein etc. CM had the PELPK mutations to a lesser extent (73% vs 29%, P < 0.05). Sequence changes in the PELPK binding motif are found in the CTT that prevent binding to CEAR and do not allow activation of Kupffer cells and thus secretion of pro-inflammatory cytokines.

This could account for the high CEA serum levels in the CTT as compared to CM and humans and contribute to the lack of liver metastasis. Zimmer and Thomas in 2001 showed in a minority of patients with advanced colon cancer all of whom had high CEA levels, changes in the PELPK sequence. CEA from these patients could not bind to Kupffer cells. These patients did not have liver metastasis though peritoneal carcinomatosis was common. Serendipitously: (2) Normal sections of human livers bearing metastatic disease (our intended “negative” controls) showed translocation of the BGP to the cytosol of the hepatocyte whereas CTT livers did not have detectable BGP except in the gallbladder wall. BGP is usually a structural protein found in the bile canalculus but conceivably, if translocated to the cytosol, may act as a downstream mediator of VEGF, facilitating the progression of liver metastasis. Human cirrhotic and hepatitis livers demonstrated orthotopic BGP compared to human livers bearing metastases (P < 0.02) where the BGP had translocated to the cytoplasm (Figure 5).

This may answer the age-old clinical question as to why patients with cirrhosis rarely get hepatic metastases from extrahepatic primary cancers. We also observed: (3) a diminution of CEAR staining (Figure 6) in the CTT liver sections compared to humans (P < 0.02), a further barrier to CEA hepatic uptake in the CTT as compared to the human liver. Surprisingly: (4) Extremely robust fibulin-5 hepatocyte labeling was detected in CTT livers (Figure 7) and finally: (5) Minimal glycosylation of CTT CEA was demonstrated as opposed to human CEA. 80% of CTT vs 60% of CM were positive for fibulin-5 binding with functions of the various fibulins contrasted in Table 3.

Hepatocytes were the predominant cell types expressing fibulin-5 in both types of animals but tended to be more intense in the CTT (0.75 ± 0.289 and 0.4 ± 0.418 respectively) and also seen to be of the same distribution and intensity in the human liver controls. Kupffer cells did not stain (P = 0.033 CTT and P = 0.09 CM) but occasional bile duct cells were

Table 3 Biological attributes of the fibulin family

| Attribute | Fibulin-1 | Fibulin-2 | Fibulin-3 | Fibulin-4 | Fibulin-5 |
|-----------|-----------|-----------|-----------|-----------|-----------|
| Invasion of endothelium | Decrease | Increase | Decrease | Unknown | Decrease |
| Binding of tropoelastin | Moderate | High | low | Moderate | High |
| Effect on angiogenesis | Unknown | Unknown | Reduced | Unknown | Reduction |
| MMP/ | Increase | Unknown | Reduced | Unknown | Reduced |
| fibronectin | | | | | |
| change | | | | | |

Table 3  Biological attributes of the fibulin family[51-53]
positive but of lesser intensity in the CTT (0.125 ± 0.25 and 0.375 ± 0.479 respectively - Figure 8). Vascular staining was seen only in 2/5 CTT sections at a mean intensity of (0.3 ± 0.447). No appreciable uptake was noted in white blood cells in CTT and minimal in CM. We therefore postulate 5 possible pathways of spontaneous hepatic metastasis evasion in the CTT, 4 of which we have previously reported [8], summarized in Table 4.

OTHER POTENTIAL ANTI-METASTATIC AND METASTOGENIC MOIETIES IN THE CTT

Lastly using the Gelco Diagnostics antibody kit to POA we determined a low level of POA [79] in extracts from CTT non-cancerous tissues 1.603 ± 1.656 µg/mL (mean ± SD). In contrast to serum in 13 humans with pancreatic cancer (9.622 ± 6.424) these levels were modest. Since normal serum levels in humans are < 15 U/mL even an order of magnitude increase in the CTT is unlikely to increase levels where they might
influence the tumor invasiveness. In the study cited, a significant correlation was found between POA and CEA levels classified by stage of CRC \((P = 0.003)\). Although further study is required it would appear that POA is not a major determinant in the CTT of the metastatic process to the extent that it is in humans making this another potential discouraging factor for the development of hepatic metastasis.

Ultimately, we would need to show that the cytokine environment milieu is similar in the CTT as described above for humans. Wilson et al.\(^{[80]}\) using 32 monoclonal antibodies has shown MHC Class I and II proteins on lymphocytic B cells (CD20-21, 23) and CD16-56 on NK cells; CD2 and CD3 and CD4/8 helper T cells. In addition IL-2 receptor CD25 receptor is present as well as B chain LFA-1 CD 18. There was however poor reactivity for CD11a; and ICAM-1 (CD54) was negative. 14 years later with more reagents available, Kap et al.\(^{[81]}\) confirmed much of the above findings with IL1, 2, 4, 6, 10, 12, and 17a found. In addition they found all 4 clones against CD11a to be positive in contrast to the above paper. CD11a and CD18 and CD29 are important factors for adhesion and are expressed in the CTT. In addition, CD40 of the TNF family is present thus demonstrating that the CTT has almost all the necessary repertoire of proteins as is present in humans to develop hepatic metastasis. Although ICAM-1 was not included in the repertoire, by virtue of CTT colitis to respond to an anti-integrin monoclonal\(^{[82]}\), we can conclude that selectins adhesion molecules are also likely expressed. This being the case, it would appear that the aforementioned inhibitory mechanisms are sufficient to abrogate this phenomenon almost completely.

We cannot rule out additional mechanisms and would encourage such research efforts. While ICAM1 may not have been confirmed in the above review (because it does not seem that it was included in the battery of monoclonals described above), E-selectin and integrins certainly have been convincingly demonstrated to be actionable by therapeutic intervention in the CTT\(^{[80-82]}\).

Certainly, the above hypotheses are not established scientific facts but they are testable and applicable to a particularly vexing problem presented by hepatic metastases. The ability to intervene using novel approaches based on these ideas will constitute the final section of this review and may represent an encouraging future vision of hope for myriads of “terminal” cancer patients.

In quoting the final chapter of the late Neal K. Clapp’s book on this topic: "Our research opportunities using the cotton-top tamarins to study colonic diseases are truly only limited by our ingenuity”.

### HOW MIGHT WE EXPLOIT THESE ADVANCES FOR THE BETTERMENT OF THE CANCER PATIENT?

Hepatic metastasis of CRC is a very common clinical situation in oncology with prevalence at the time of diagnosis of approximately 20%-25%. The liver has been shown to be the most common site of metastatic spread of colorectal cancer. The prognosis of colorectal hepatic metastases has improved in the last few years owing to surgical resection of liver metastases for patients with no extrahepatic disease; however, liver metastases are resectable in only 15% of the cases\(^{[83,84]}\). Often, factors such as location, size and number of hepatic metastases make it difficult to resect these tumors\(^{[83,85]}\). Thus early prevention of metastasis is crucial in improving the prognosis of patients diagnosed with primary colorectal cancer. As earlier documented by Smedsroed et al.\(^{[86]}\) colorectal hepatic metastasis involve four steps which are interrelated; the cancer cells must establish micrometastasis in the liver by passing through microvasculature which is guarded by Kupffer acting in concert with NK cells. To ensure nutritional supply and establish clinically evident metastatic disease, these cells engage growth factors and adhesion molecules such as VEGF, VCAMs etc. As can be appreciated from both clinical and experimental evidence, Kupffer cells are involved with all the rate limiting step of this macrometastatic disease process.
Gellan gum is an exocellular microbial polysaccharide consisting of repeating tetrasaccharide units of glucose, glucuronic acid and rhamnose in a molar ratio of 2:1:1. It has a wide range of applications in the pharmaceutical industry. In this area, its use has been mainly concentrated in ophthalmic drug delivery and oral sustained-release preparations, in which its ability to undergo cation-induced gelation is utilized as the main fabrication platform. Gellan gum has a number of advantages as it can undergo temperature-dependent as well as cation-induced gelation, which may be of importance for in vivo slow release of encapsulated gadolinium. It also has proven non-toxicity in humans. The FDA has also approved gellan gum as a food additive for human consumption, thus, its safety in humans is established. To improve the effectiveness of this targeting system, a three-dimensional matrix via covalent crosslinking with an end-reactive polyethylene glycol (PEG) through amide linkage can be used. Such modification provides a stronger gellan gum matrix that is nontoxic to normal cells and possesses the ability to deliver biological molecules such as gadolinium to target cells in a controlled manner as illustrated in Figure 10. There is some experimental animal model work that suggests that gadolinium in the form of metallofullerol nanoparticles inhibit cancer metastases but the metastases observed were mainly pulmonary.

USE OF GADOLINIUM BLOCKADE TO DEplete kupffer cells

In our earlier study using CTT, we showed that although they have CEA receptors similar to humans, they are able to evade CRC hepatic metastasis via two possible means; defective CEA receptors with much reduced receptor capacity or by sequence changes in the PELPK binding motif that is required for receptor activation (Table 2). We hypothesize exploiting methods to prevent CEA/CEAR binding in humans may prove effective in reducing the occurrence of hepatic and possibly lung metastasis. This hypothesis was tested earlier using gellan gum to block uptake of CEA by sinusoidal cells. Although the result was promising, only a transient blockage of CEA was achieved mostly due to the short wash out period for gadolinium. Therefore, using animal models this approach can be refined to optimize our earlier findings using gellan gum as blocking agent for Kupffer cells and the CEA/CEAR interaction.

As a novel approach to anti-metastatic therapy, gadolinium may be encapsulated in smart nanodevices that will prolong the residence time significantly. Using PEGylated-gellan-gum as the polymer of choice, gadolinium can be encapsulated in nanoparticles with average size of 250 nm. Gellan gum is an exocellular microbial polysaccharide with a natural propensity to absorb biological fluid in vivo thus regulating the rate of drug/agent release. It consists of repeating tetrasaccharide units of glucose, glucuronic acid and rhamnose in a molar ratio of 2:1:1. In addition to being used as a food additive, gellan gum has a wide range of applications in the

Tobi M et al. How the CTT avoids liver metastasis

FIBULIN AS A TREATMENT FOR HEPATIC METASTASIS

The process of nanoprecipitation described by the schematics in Figure 10 can potentially be applied to protein encapsulation provided the formulation parameters are optimized as described earlier by our group. Fibulin-5 plays an important role in antagonizing angiogenesis in humans; furthermore its overexpression has been shown to inhibit HCC cell migration and invasion in vitro, thus fibulin-5 could also be encapsulated in gellan gum nanoparticles to increase their in vivo residence time. Nanoparticles formulation should be optimized as described earlier prior to encapsulation of fibulin-5. We would expect fibulin-5 to be the most effective of the fibulins in reducing angiogenesis but fibulin-3 may also be considered (Table 3). While fibulin-5 has been shown to suppress metastasis to the liver and lung, it has yet to be used directly in a nanotechnology formulation as we propose. A report that human oncogenic fibulin-5 may promote nasopharyngeal cancer metastasis would suggest that we proceed cautiously with this approach with both agents.

TARGETING CEA/CEAR INTERACTIONS AS A THERAPEUTIC APPROACH TO LIVER METASTASIS

CEA has been shown to be a mediator of metastasis for colorectal cancer by causing changes in the tumor microenvironment that increase the potential for tumor cell implantation and growth. CEA interaction with
its receptor therefore suggests a targeted approach to therapy. A number of methods could be used to achieve disruption of CEA/CEAR including the use of antibodies specific for CEA or its receptor. In general this type of approach has worked well against various cancers and growth receptors e.g., EGFR. Other approaches using small molecule inhibitors of ligand receptor interactions are also being used, In particular RNA aptamers hold great promise as therapeutic agents. These aptamers are now being used in studies involving CEA. Lee et al have described the use of a RNA aptamer directed against the PELPK motif of CEA to prevent liver metastasis from the mucinous colon cancer cell line LS174T. They suggest this occurred by disruption of CEA/CEAR interaction. LS174T produces large amounts of CEA and has a high metastatic potential to the liver. Recently, aptamers have also been used against the homotypic adhesion sites of CEA to block tumor cell/cell interactions further emphasizing the feasibility of this approach.

Aptamers are also being used for radiolocalization of CEA producing tumors and may even supplant the use of antibodies for this purpose. The use of CEA antagonists to reduce the metastatic potential has now become feasible especially with the advent of small molecule inhibitors. More pre-clinical trials are needed before these approaches can be used in cancer patients at high risk for metastases.

CONCLUSION

We thus have the means to potentially interfere with the uptake of CEA from the hepatic sinusoid Kupffer cell receptor and hopefully disrupt the inflammatory cascade and well as intervening in the fibulin pathways in delivery of fibulin-5 to shore up hepatic defenses against tumor spread to the liver. In time, additional interventions will be designed to make this a reality.
based on the lessons learned from the cotton top tamarin- the artful liver metastasis dodger.

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