Mouse Models of Colorectal Cancer and Liver Metastases

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Introduction

With 940,000 recorded cases worldwide per year, colorectal cancer (CRC) is the third most common malignancy in the world. Despite efforts to improve prevention and therapy, nearly 500,000 patients die from CRC each year [1]. It has become evident that the main problem in the treatment of CRC is not so much eradication of the primary tumor, but rather the formation of incurable metastases. Mortality is particularly associated with the occurrence of metastases in the liver.

Animal models for human CRC can provide insight into the mechanisms that underlie the development and pathogenesis of CRC. The ideal animal model should therefore faithfully replicate all aspects of tumor development in man. These include the (sequential) acquirement of genetic alterations with consequent changes in cell behavior and tumor biology. In addition, the metastatic potential and the characteristic sensitivity to therapeutics should ideally be conserved [2]. Furthermore, the tumor model should be practical: tumor take should be predictable and consistent, with a high incidence of affected animals in a narrow time frame [3]. Unfortunately, although a number of models approximate some of the characteristics of human CRC, none meet all these criteria. Therefore, each specific experimental issue should be studied by choosing the model best suited to resolve that particular issue. This review highlights the mouse models cur-
Mouse models of spontaneous and carcinogen-induced CRC are the earliest models described. Spontaneous formation of colorectal cancer in mice occurs with an incidence of <1% [4, 5] making it impossible to perform experimental studies on CRC development. Although inbred CRC-prone mice develop the disease at a young age, the predictability and reproducibility of tumor take are insufficient for routine experimental use. In addition, the formation of metastases occurs only sporadically in these mice [6].

In order to increase the incidence of CRC, new models were developed in which mice are exposed to carcinogens. Most carcinogens cause malignancies in multiple organs, but some predominantly induce CRC. Frequently used carcinogens are dimethylhydrazine (DMH) and its metabolite azoxymethane (AOM), N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and N-methyl-N-nitrosourea (MNU) [6]. The incidence of CRC development depends on the carcinogen used, the dosage, the duration and frequency of administration, as well as on the timing of administration. DMH and AOM can be administered via four routes: oral, subcutaneous, intrarectal and intramuscular. The effectiveness of various carcinogens in inducing colorectal cancer has been compared [6]. Oral dosing of DMH in rats results in a low tumor incidence (14 to 30%). Subcutaneous administration of DMH or AOM results in a colon tumor incidence varying from 0 to 100%. Intrarectal administration of DMH induces mild hyperplasia and preneoplastic lesions in the colon after 34 weeks. Repeated intramuscular injections of AOM resulted in 80% incidence of CRC after 12 weeks. Oral dosing of direct-acting MNU induces tumors along the entire gastrointestinal tract, with a decreasing incidence from stomach to rectum. Intrarectal administration of MNU resulted in a colon tumor incidence of 100%. Moreover, metastases developed in 23 to 31% of mice [7]. In addition, the sex, age and genetic background of the mice affect the incidence of CRC development. Moreover, the intestinal flora, the diet and the immunological status of the mice can interfere with the metabolism of carcinogenic compounds and thereby influence their effective local concentration.

Ideally, all of these variables should be standardized in order to obtain a protocol with which CRC development can be reproducibly induced. A major disadvantage of carcinogen-induced CRC development is the low incidence of tumor take, making it necessary to use large numbers of animals. Moreover, these models are not suitable for studying metastasis formation as this occurs very slowly and infrequently. However, carcinogen-induced CRC development in mice is ideal for studying the influence of diet on tumor development.

Molecular Genesis of Colorectal Cancer

Multistage carcinogenesis from local hyperplasia to adenoma to invasive carcinoma and metastatic disease requires the sequential mutation of various genes. Four classes of genes are of importance in carcinogenesis: the growth-promoting proto-oncogenes (dominant), the growth-inhibiting cancer-suppressor genes (recessive), genes that regulate apoptosis (dominant or recessive) and genes that regulate repair of damaged DNA. The DNA repair genes affect cell survival or proliferation indirectly by influencing the ability to repair nonlethal damage in other genes, including the proto-oncogenes and the tumor-suppressor genes. A defect in the DNA repair genes predisposes to mutations in the genome and hence to neoplastic transformation.

‘Gatekeeper’ genes directly influence the growth of cells. Mutations in these genes can initiate the entry into the multistep carcinogenesis pathway. In contrast, ‘caretaker’ genes affect genomic stability. The mismatch repair genes belong to the latter category.

Although most colorectal cancers in human are regarded as sporadic, a small percentage is due to an autosomal-dominant inherited syndrome [1]. These patients carry a germ-line mutation of either a gatekeeper or a caretaker gene; therefore, they have an increased risk of developing cancer. Hereditary nonpolyposis colon cancer (HNPCC) is the most common form of hereditary colon cancer, accounting for 5–8% of all colon cancers [8]. Colon cancer develops as a result of mutations in a caretaker gene. Familial adenomatous polyposis (FAP) accounts for less than 1% of all colon cancers [9]. As a result of a mutation in a gatekeeper gene multiple adenomas develop in the intestinal tract, which eventually progress to malignant colon tumors.
Mouse Models for HNPCC

Hereditary non-polyposis coli (HNPCC) is a syndrome caused by the inactivation of DNA mismatch repair genes (e.g. MLH-1, MSH-2 and -6, PMS-1 and -2). The tumors are characterized by instability at short tandem repeat sequences, also called microsatellites. Models based on the use of genetically modified mice have contributed greatly to our understanding of the complicated process of tumour development. Mouse models have given insight into the mechanisms underlying the development of CRC. Mice homozygous for mismatch repair (MMR) genes and heterozygous for a defect in the gatekeeper gene APC have shown that the MMR gene enhances APC-mediated intestinal carcinogenesis. Exposure of MMR-deficient cells to endogenous or exogenous mutagens may potentiate tumorigenesis and may be critical in the organ selection in HNPCC [10, 11]. These models also revealed that MMR-deficient cells fail to induce apoptosis in response to alkylating agents. In contrast, the alkylated base damage remains in the DNA thereby potentiating carcinogenesis. This may have direct implications for chemotherapy of HNPCC patients [14].

Mouse Models for FAP

As mentioned before, a sequence of specific genetic changes underlies the development of intestinal carcinogenesis in the mouse. These include inactivation of the tumor suppressor genes APC, p53 and Smad3 and activation of the proto-oncogene K-Ras. Mice carrying specific deletions of the APC gene develop multiple adenomas throughout the gastrointestinal tract, especially in the small intestine [15], but there is no tumor development in the colon. In other models, for example the Multiple intestinal neoplasia (Min) mouse models, additional somatic changes need to be induced by applying carcinogens for high incidence intestinal tumor development. Mice heterozygous for the APC gene have been used to study the general principles of carcinogenesis, to test the response to suppressive agents such as aspirin [16, 17] and to test the carcinogenicity of various compounds [18].

Inactivation of the p53 gene mainly leads to lymphomagenesis, although colon tumors can develop after administration of colon-specific carcinogens [6, 19]. Whereas the APC mice can be used for studying the early stages of intestinal polyp formation, the p53 knockout mouse is particularly suited for studying the influence of diet, carcinogens and chemotherapeutics on tumor progression. Studies in p53-deficient mice have revealed that absence of p53 interferes with the physiology of apoptotic cell death, i.e. cell cycle arrest after DNA damage is failing. It has been shown that several chemotherapeutics, such as ectopiside, adriamycin and 5-fluorouracil, can induce p53-mediated apoptosis. Tumors that retained wild-type p53 were responsive to these therapies, in contrast to tumors derived from p53 null cells. These findings indicate that human cancer patients with p53 mutations in a tumor might be resistant to these therapies [20].

TGFβ-related growth factors have also been implicated to play a role in tumor formation. TGFβ transduces its signal into the cell via the second messengers Smad2, Smad3 and Smad4. It has recently been shown that Smad3 mutant mice develop metastatic colorectal cancer. All inbred Smad3−/− mutants had colorectal adenocarcinomas, some of which were highly aggressive and approximately one-third invaded through all layers of the bowel wall. In several mutant mice lymph nodes were enlarged and showed infiltration by carcinoma [21].

Conditional Gene (In)Activation to Model FAP

Germline transmission of disrupted tumor suppressor genes or activated oncogenes can create cancer-prone mice. Such mice have proven to be powerful tools for studying gene function in tumor biology. For example, the different APC mutant mice faithfully model familial adenomatous polyposis coli, but are less suitable for studying sporadic CRC, as the initiating mutation is transmitted through the germline. In addition, tumors may develop too early for experimental therapeutics, causing high mortality rates at a young age. New techniques have made it possible to induce time-controlled and tissue-specific somatic gene activation or inactivation [22–28]. An example of such a model is the ApcloxP mouse in which exon 14 of the APC gene is flanked by...
The adenomas that develop in the mouse models described above show proper resemblance to the equivalent lesions in FAP patients. Furthermore, the (mostly intestinal) tumors form spontaneously, predictably and with a high incidence. They are nonimmunogenic and arise in immunocompetent mice (in contrast to the implantation models described later). A disadvantage is that metastases form infrequently (if at all) and unpredictably. However, if they form, they do so spontaneously thereby resembling the process in cancer patients [32].

Advantages, Limitations and Applications of the Genetically Engineered Mouse Models

The genetically engineered mouse models are used primarily to determine whether a particular gene is involved in the pathogenesis of cancer, or whether a gene product, involved in specific signalling processes, contributes to cancer in combination with other predisposing conditions. Because the development of colorectal cancer in these models is not predictable and metastases seldom develop, they are not often used in drug testing [3]. Despite their shortcomings, such models can be used to study the effects of early therapeutic intervention or preventive measures. Such studies are likely to be predictive of the clinical outcome, because they represent the natural history of tumour development. For example, a comparison was made between the efficacy of agents on the prevention of CRC in mice and polyp recurrence in humans [18]. NSAIDs strongly decrease the tumor yield in the small intestine of Min mice. This is consistent with epidemiological studies suggesting that NSAIDs might decrease the colorectal cancer incidence in humans.

With the development of the conditional gene (in)activation models it has become possible to generate highly specific tumors in a narrow time window with high incidence [26]. These models provide new opportunities to establish the influence of specific (combinations of) genes in the initiation and progression of cancer. Conditional mouse models will be ideally suited to study drugs that interfere with specifically mutated regulatory pathways promoting tumor growth [26]. The potential of this approach can be illustrated by an experiment in which tumor cells with amplified c-myc (an oncogene) expression and wild-type p53 are susceptible to 5-FU, in contrast to tumor cells with a mutated p53 gene. In accordance with these findings, retrospective analysis of a phase III clinical trial showed that only patients with tumors containing amplified expression of c-myc and p53 responded to therapy with 5-FU [33].

Disadvantages of these models are insufficient expression in the presence of the inducer (poor inducibility) or unwanted expression in the absence of the inducer (leakiness). As a result, expression of the target gene may not be 100% tissue specific [24].

Implantation Models

Grafts from either human (xenografts) or murine (allografts) tumors can be implanted into recipient mice. To prevent xenograft rejection, either nude mice or mice with severe combined immunodeficiency (SCID) are used. In nude mice the Ns gene is knocked out, resulting in hairless thymus-less mice which cannot generate T lymphocytes. SCID mice have a mutation in the cytokine (IL-2) receptor resulting in disrupted lymphocyte matura-

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heterogeneous nature of the material, both within a tumor, as well as between different tumors. This makes standardization and comparison between various experiments difficult. Furthermore, human tumor tissue often contains large necrotic areas that are not suitable for experimentation [37].

**Ectopic and Orthotopic Tumor Models**

There are several methods for implanting human tumor cells or tumor tissue (table 1). By far the easiest and most frequently used model is subcutaneous injection/implantation. The accessibility of subcutaneous tumors is a tremendous advantage for monitoring tumor progression and for assessing the effects of therapeutic intervention. However, a major disadvantage is that the subcutaneous (ectopic) microenvironment greatly differs from that of the colon or the liver. Interactions between the host environment and the tumor graft determine tumor cell expression profiles, the levels of growth factors and nutrients, as well as tumor angiogenesis and metastatic behavior. For instance, CRC growth in the colon will give rise to metastases, whereas subcutaneous growth does not [35, 37, 39–41].

Alternatively, tumor growth at orthotopic sites (i.e. the colon, the cecum or the rectal wall) can be achieved by subserosal injection of tumor cells, or by surgical orthotopic implantation (SOI) of tumor fragments. These fragments (typically 1 mm³) can be obtained either from biopsies or from previously grown tumors and are sutured onto the cecum, the colon or the rectal wall. For successful attachment of the implant, it is recommended to damage the serosal lining of the intestinal wall using a surgical blade prior to implantation. All of these procedures are carried out with the aid of a microscope.

Orthotopic and ectopic organ environments differentially influence the sensitivity of tumor cells to chemotherapeutics [37]. For example, colon cancers grown subcutaneously were more susceptible to doxorubicin than tumors growing in the cecum of mice [42].

**Models for Liver Metastasis Formation**

The preferred site of metastasis formation in CRC patients is the liver. Because any therapy of cancer is invariably the therapy of metastatic disease, it is essential that models in which CRC actually forms, (liver) metastases develop as well. Surgical orthotopic implantation of highly metastatic tumor cells in the colon of nude mice has been shown to result in the rapid and efficient formation of metastases in liver (100%), lymph nodes and spleen [35, 36, 43–45]. This model is ideal for studying many aspects of metastasis formation as well as for analyzing the efficacy of novel therapeutic agents. Liver metastases in the mouse can also be induced by intrasplenic or direct intraportal injection of colon cancer cells. As the tumor cells reach the hepatic microcirculation, they become arrested either by size restriction or by specific retention. Arrested cells may grow out while still within the vascular or they may extravasate and colonize the liver parenchyma. Although a small percentage of injected cells eventually manage to grow out and form local tumors, the overall efficiency of (liver) metastasis formation is low.

The metastatic potential of a given tumor graft or a cell line can be augmented by so-called ‘passage cycles’. During this procedure the liver metastases are isolated, expanded in vitro and are subsequently re-injected into the spleen or the vena porta. The resulting liver metastases can then be re-isolated. With each selection cycle the metastatic potential of the cells increases [46]. This also means that the cell populations selected by this procedure have become genetically distinct from the original cell line and should be treated as independent subclones.

The above techniques result in the colonization of the entire liver with single tumor cells. However, for some types of experiments, it may be required to grow tumors in a single liver lobe or even as a solitary tumor. To obtain tumor growth in an isolated liver lobe we usually perform subcapsular injection of tumor cell suspensions. A single liver tumor may be obtained by implantation of tumor fragments from pre-established (subcutaneous) tumors. During this procedure, a small incision in the liver parenchyma is made, followed by rapid implantation of the fragment. This usually stops most of the bleeding, but further hemostasis may be achieved by applying gentle pressure at the wound surface using cotton. For example,
this intrahepatic model has been used to study the efficacy of 5-fluorocytosine/cytosine deaminase enzyme prodrug gene therapy. Cells expressing cytosine deaminase activate the prodrug 5-FC into the cytotoxic agent 5-FU. This approach has shown to inhibit intrahepatic CRC tumor progression [47].

A major disadvantage of the experimental metastasis models is that only late stages of metastasis formation can be studied. The early stages, including local invasion at the site of the primary tumor and gaining access to lymphatics or blood vessels, are by-passed by direct injection of tumor cells into the portal vein or into the spleen. Proper modeling of the early phases of spontaneous metastasis formation requires growing a metastatic tumor orthotopically (i.e. in the colon). A disadvantage of this method is the poor efficiency and predictability of spontaneous metastasis formation from an orthotopic primary tumor. Nonetheless, models involving spontaneous metastasis formation have been used to test the efficacy of experimental therapeutics in preventing metastasis. Hepatotropic liposomal adriamycin (hLip-ADM) has been shown to effectively inhibit liver metastasis (0 vs. 83% in control animals) from orthotopically implanted colon cancer [48]. As an alternative method to study early metastasis formation, noninvasive imaging techniques may be used to follow intraportally injected cells through the early phases of cell arrest, extravasation, colonization and growth (see below).

Advantages, Limitations and Applications of the Implantation Models

The advantages of the implantation models are the high degree of predictability and rapidity of tumor formation. The major advantage of studying s.c. growing tumors is the possibility to screen large numbers of drugs. The models require minimal labor, are relatively inexpensive, rapid and reproducible. Limitations are the inefficient formation of metastases and the danger of scoring false-positive results when testing anticancer drugs in experimental therapies. Tumors growing subcutaneously may show unnatural reactions to experimental drugs [37, 42], possibly because the expression profile of multidrug resistance (mdr) genes is altered [49, 50]. Thus, therapeutic results obtained in a subcutaneous tumor model should always be followed by studies in an orthotopic implantation model, preferably one in which metastases develop [51].

Drugs that modulate the tumor microenvironment cannot be evaluated by ectopic models and must be studied in orthotopic models. Compounds that activate the immune system should be tested in immunocompetent mice. Furthermore, it is necessary to standardize protocols with respect to the route, the method and the dosing of tumor cell inoculation, as well as that of the therapeutic agents.

The applicability of the various mouse models described is summarized in table 2.

Imaging and Monitoring Tumor Growth and Metastasis Formation

Histological and Molecular Profiling

To what extent do CRC tumors in mouse models resemble the tumors in CRC patients? Several modern molecular biological techniques may be applied to answer this question. It is expected that the analyses of gene expression and protein abundance profiles will become extremely important in tumor typing for diagnostic purposes. These techniques are also best suited for comparing human tumor behavior in patients and in mice. Techniques that have been used to date include comparisons of histological and chromosomal parameters [52].

Histological analyses can also be applied for quantifying tumor growth postmortem. The outgrowth of CRC liver metastases is routinely determined by measuring the area of liver tissue that has been replaced by tumor tissue. Histological analysis of tumor growth has two major disadvantages. First, analyses can only be performed once, postmortem. Second, it is time-consuming and laborious. These two disadvantages can be overcome by using modern noninvasive imaging techniques.

Noninvasive Imaging Techniques

Noninvasive imaging techniques allow monitoring of tumor initiation, progression and response to therapy in a single animal by sequential imaging analysis. Conventional imaging methods that are used in the clinic have been adjusted to visualize murine tumors [53, 54] including magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET) and single photon emission tomography (SPECT). Furthermore, fluorescence and bioluminescence imaging techniques allow for monitoring the growth of tumors expressing either Aequorea victoria green fluorescent protein (GFP) or firefly luciferase.

EGFP and Fluorescence Imaging

Ultrasensitive charge-coupled device (CCD) cameras can detect tumor cells that continuously express EGFP at

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high concentrations. The development of CRC liver metastases has been imaged noninvasively by this method [55–57]. One of the drawbacks of this technique is that the high level E-GFP expression that is required for noninvasive imaging may interfere with the growth and survival of a number of CRC tumor cell lines. Another limitation of using fluorescence imaging based on E-GFP expression remains the difficulty of detecting and quantifying the fluorescence emitted from GFP-expressing tumors through the abdominal wall, due to scattering and light absorption. The latter problem can largely be prevented by creating a flap of skin that may be opened whilst imaging and closed afterwards for multiple measurements of tumor growth in a single mouse. Alternatively, the tumor-bearing tissue can be exposed surgically and in this way it is possible to visualize local tumor-tissue interaction, tumor progression, angiogenesis and metastasis formation at the single cell level. Although technically possible, ethical considerations make it undesirable to perform multiple invasive imaging analyses on a single mouse. Despite the disadvantages outlined above it is expected that with modern laser scanning technologies and improved detection limits and signal-to-noise ratios, it will be possible to follow the growth of (auto)fluorescent tumors noninvasively on a routine basis.

Luciferase and Bioluminescence Imaging
An alternative to following tumor growth by fluorescence is to make use of bioluminescence. To this end, tumor cells must be engineered to express luciferase, a photoprotein that generates light following oxidative conversion of its substrate luciferin. A disadvantage of this system is that prior to imaging, the mice must receive an intraperitoneal injection of luciferin. Low-light photon-counting cameras can subsequently detect the light emitted from the tumor cells (fig. 1). This technique is ideal for visualizing tumor growth in a noninvasive and sequential manner and it has successfully been used to study the growth of CRC cells in the mouse liver (fig. 2). Moreover, chemotherapeutic effects on intrahepatic CRC were easily assessed by this method [47].

In conclusion, optical imaging (bioluminescence and fluorescence) allows monitoring of tumor growth, progression and metastasis formation as well as the response

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of the tumor to therapy. When compared to classical (two-dimensional) histological analyses, the imaging techniques show improved (three-dimensional) accuracy and reproducibility as well as the enormous advantage of serial measurements. Fewer animals are therefore required to collect statistically reliable results [26].

Additional Considerations in Modeling Cancer in Mice

There are few notorious differences between mice and human and confounding variables that influence carcinogenesis. These differences make accurate modeling of cancer difficult and careful interpretation of the mouse models is therefore essential.

An important issue is telomeres, structures at the chromosome ends that protect chromosomes against damage. Telomere exhaustion results in apoptosis [58]. Telomerase activity prevents telomere shortening, thereby allowing immortal growth of cells. Humans do not have functional telomerase in their cells, in contrast to mice. Through knockout of the gene encoding the telomerase enzyme (Terc−/− mice), mouse models with more human-sized telomeres can be created [59].

Another point meriting attention is age-dependent carcinogenesis. Cancer is predominantly a disease of the elderly. In contrast, cells divide less frequently in older mice, which probably makes the mice less susceptible to tumor initiation and progression. A study in a Min/+ mouse model revealed that younger mice were more sus-
ceptible to AOM-induced colorectal carcinogenesis than older mice [60].

Dietary factors and physical activity may also influence carcinogenesis. Dietary components may be carcinogenic [61], or may improve cellular defense mechanisms [62]. Dietary restriction may have an anticarcinogenic effect [63], and limited physical activity might increase the incidence of colorectal malignancies [64].

For adequate interpretation and comparability it is crucial to standardize these factors. By doing so, existing differences between the many different laboratories may be diminished.

Conclusions

The ideal animal model does not yet exist, although a mouse model can imitate parts of the human carcinogenesis. Mouse models can only be used as predictors of outcome. Therefore, it is crucial to use the optimal model to resolve a specific experimental question (table 2).

Chemical-induced models can be used to study dietary influences on carcinogenesis. The genetically engineered mouse models can be applied to study the influence of genetic changes on carcinogenesis and the interaction of these mutations with environmental factors. The ectopic implantation models can be used to screen a large number of cytotoxic agents. To verify the effects of experimental therapies found in ectopic mouse models, to evaluate drugs adjusting the tumor-host interaction, to study therapy for metastasis and to analyze the pathogenesis of metastases, the orthotopic implantation models are necessary. Syngeneic implantation models are suitable for immune therapy experiments.

Standardization of protocols used and adequate description of techniques used is equally important, in order to make comparison between various experiments and laboratories possible. Novel imaging and molecular tools will further improve efficacy, accuracy and reproducibility of mouse models. By combining the (latest) mouse models with sophisticated imaging technologies it might be possible to accelerate the development of colorectal cancer therapies.

References

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