

IN-VIVO AND IN-VITRO MODELS ON COLON CANCER

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ABSTRACT

In-Vivo and In-Vitro models are thought to be the best model's for to obtain transferable results. The present review aims to give a short overview of the existing in-vivo and in-vitro models and their characteristics. Four different autochthonous tumor models; 1,2-dimethylhydrazine, N-methylnitrosourea, N-methyl-N'-nitro-N-nitrosoguanidine, N-nitroso-acetoxymethyl-methylamine, are the In-Vivo models and the In-vitro models; studies involving primary cell cultures, studies on cell line, Brine Shrimp Lethality Bioassay, MTT (Microculture Tetrazolium) assay, SRB (Sulphorhodamine B) assay, Trypan Blue Exclusion and Crystal Violet Inclusion these are used worldwide for cell viability, cell growth studies of colon cancer cell line. The choice of the model for experimental investigation of colon cancer should be depends on the clinical equations.[5]

KEY WORDS

In-Vivo and In-Vitro models-Experimental colorectal carcinoma.

INTRODUCTION

Colon cancer is one of the most common malignancies in many regions of the world is thought to arise from the accumulation of mutations in a single epithelial cell of the colon and rectum. The idea that this cancer might be a root cause for chemoprevention stems from epidemiological evidence that some factors in the diet may play important roles in its development, where others may reduce the risk. Experimental Colon carcinogenesis is a multistep process involving three distinct stages, initiation, that alters the molecular message of a normal cell, followed by promotion and progression that ultimately ends up with a phenotypically altered "transformed cell". In animal studies, treated with a carcinogen, such as, 1,2 dimethylhydrazine (DMH), methylnitrosourea, N-methyl-N'-nitro-N-nitrosoguanidine will induce colon tumors in experimental animals

particularly in rats and for invitro studies such as, MTT Assay, SRB Assay, Trypan Blue Exclusion Assay requires different cell line.

IN -VIVO TESTING

Human cancer is autochthonic (Chemical induced) with the exception of choriocarcinoma and tumors transplanted with an organ from a cancer patients. It seems to that tumors in animals equal in their genesis to those in man are also comparable in biology chemosensitivity. It is accepted today that exogenous chemicals induce most of human cancer.

Autochthonic means remaining at the site of formation. A blood clot that has not been carried in the bloodstream from its point of origin. Using this autochthonic model not only biology of cancer but also the etiology of these tumors can be studied. Today there is some evidence that autochthonic tumor models can give more

precise answer on questions concerning the induction, development and treatment of cancer than transplantation models on cancers.

Criteria for autochthonic animal tumors as a model for chemotherapeutics studies [2].

- ✓ Unilocular tumor occurrence.
- ✓ Short, well defined collective tumor induction period, with little individual deviation.
- ✓ Tumors diagnosable in time.
- ✓ Tumor biology should parallel human counterparts.
- ✓ Chemosensitivity should parallel human counterparts.

AUTOCHTHONIC COLORECTAL CANCER MODELS

A. First autochthonic tumor model: 1,2 Dimethylhydrazine:

1, 2 Dimethylhydrazine (DMH) is very effective and selective carcinogen of the colon following subcutaneous application in BD rats.

DMH is easily oxygenated to azomethane (AM) and azoxymethane (AOM) utilizing the same degradation pathway for all three substances. By oxidative dealkylation from AOM to methylazoxy methanol (MAM) it enters the degradation pathway of cycasin. The final alkylating carcinogen is produced by further metabolism of MAM and can be identified as a diazonium compound. [3]

Organ manifestation and tumor distribution depend highly on the dose of the carcinogen. The optimal dosage for the selective induction of colonic tumors is 10 mg/kg for 25 weeks (total dose = 250 mg) [4]. The time of induction is

between 180-240 days with a tumor rate of 90%. This includes all localizations. At a higher dosage of DMH the incidence of tumors beside the colon increases.

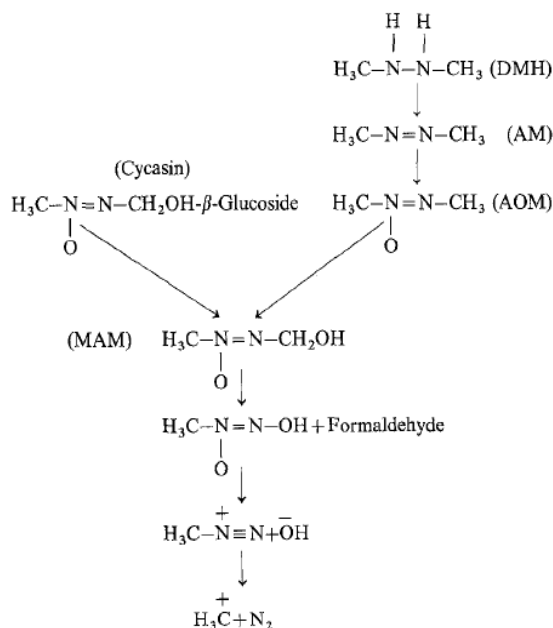
The DMH model also leads to a high tumor spread (93%) in contrast to other autochthonous tumors. An important disadvantage is the high incidence of primary tumors in other organs. Because of tumor localization in the whole colon control of tumor size and growth is difficult. DMH-induced colon carcinoma in the rat is comparable to human colon cancer. Today it is the model most used to study colon cancer.

B. Second autochthonous tumor model: N-methylnitrosourea:

N-methylnitrosourea (MNU) induces tumors by alkylation. It has been shown that methylation of the guanine base in DNA arises from AM, which in turn comes from DMH and MNU. Also alkylation of proteins and RNA has been reported.

MNU is an exceptionally unstable substance with a very short biological half-life. Particularly following i.v. application tumors are found in many different organs (brain, bone, stomach, small bowel, colon, pancreas kidney, breast, and nervous system).

Systematic investigations for the induction of colon tumors by intrarectal application resulted in a high incidence (up to 100%) of multiple colon tumors. Histopathologically these tumors are characterized as adenocarcinomas with a high grade of invasion and low spread. The disadvantage of this model is the time consuming application often necessary. [5]



C. Third autochthonous tumor model: N-methyl-N'-nitro-N-nitrosoguanidine:

Locally instilled N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) has been shown to have a direct carcinogenic effect. MNNG is degraded in alkaline milieu without enzymatic action to the carcinogen diazomethane. This rapid degradation to the carcinogen is the cause of the local cancer induction.

After intrarectal instillation at a dose of 2 mg/kg three times a week (total dose = 150 mg/kg) tumors were observed at the instillation site between the 250th and 356th day of the trial. Tumors were found in 97% of the animals.

Tumors in other locations were not reported. The disadvantage of this model is the long period of induction and latency.[6,7]

D. Fourth autochthonous tumor model: N-nitroso-acetoxymethyl-methylamine:

Like other nitrosamines N-nitroso-acetoxymethyl-methylamine (AMMN) is not per se carcinogen but is degraded to the carcinogen diazohydroxide. Intrarectal instillation of 2 mg/kg every week for 10 weeks (total dose = 20 mg/kg) showed tumors in 97% of the treated animals. The latent time was up to 252 days. Tumor metastases were found in up to 28% of the animals.[8]

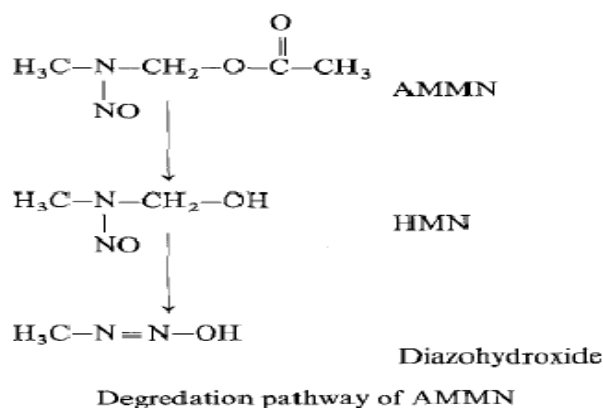


Table 1: Comparison of the four autochthonous tumor models described

| Drug | Dose | Application form | Time of induction(days) | Distribution in colon |
|---------|---------------------------|------------------|-------------------------|-----------------------|
| 1,2-DMH | 10mg/kg/week 25 weeks | S.C | 180-240 | Total |
| MNU | 3x08mg/kg/week | Intrarectal | 175-245 | Distal |
| MNNG | 3 x 2 mg/kg/week 25 weeks | Intrarectal | 230-350 | Distal |
| AMMN | 2x10mg/kg/week | Intrarectal | 210-280 | Distal |

IN VITRO TESTING:

Colorectal epithelial cells are constantly exposed to complex mixtures of compounds derived from the diet and the digestive process. These include genotoxic carcinogens and toxic or growth stimulating agents that can act as tumor promoters, but also protective compounds that counteract these effects.

While demonstration of in vivo tumor induction is the only definite proof of carcinogenic or tumor-promoting activity, the experiments are expensive and time consuming. Both for the identification of candidate compounds worth testing in vivo and for the analysis of cell biological mechanisms in vitro models are of great importance. They have to satisfy two basic requirements:

- (1) Availability and easy handling for high-throughput testing.
 - (2) Retention of tissue characteristics to support interpretation of results for the in vivo situation.
- Unfortunately tissue culture models for the target cells—namely normal and premalignant colorectal epithelial cells (CEC)—are very difficult to obtain. Therefore requirement of different cell cultures and different cell lines for in vitro studies which are given below [Carroll, 1981; Mahmoud et al., 2000].

STUDIES USING PRIMARY CULTURES:

Epithelial cells can be isolated from colorectal tissue specimen and put into primary culture.

Even cells isolated from normal mucosa that have very stringent culture requirements can be maintained for a few days. The advantage of primary cultures lies in the direct comparability: they contain the same combination of cell populations that are also present in the tissue and not sufficient time has passed for selection mechanisms to have taken effect. For human CEC (colorectal Epithelial Cells) biopsy specimen can provide material for four to five cultures, larger surgical specimen can supply sufficient material for extended studies [Buset et al., 1986].

STUDIES IN CELL LINES:

While primary cultures have provided many insights into the growth control of CECs and the effects of tumor promoters on the CECs, they are not suitable for the analysis of large groups of compounds. Neither can they easily provide sufficient material for the investigation of protein or RNA expression. For these purposes cell lines have to be used. A multitude of different carcinoma cell lines have been successfully used for studies of regulatory mechanisms and for the identification of chemopreventive compounds that induce apoptosis in the tumor cells [Willson et al., 1987][11]. HT-29 Cell lines were obtained from the primary tumor of a Caucasian woman with colon adenocarcinoma [12].

Table 2: Examples of cell line studies of tumorigenic or protective compound's in colon

| Cell Line | Test compound | Effect Observed |
|--------------------|---------------------|---------------------------------|
| SW480 | Carotene, retinoids | Inhibitor of DG-induced growth. |
| HT29, SW480 | Anthraquinone | Proliferation. |
| HT29, SW480 | Tyrphostines | Induction of apoptosis |
| HT29, SW480 | H7, calphostine | Induction of apoptosis |
| HT29, SW480 | Flavonoids | Induction of apoptosis |
| SW480, HT29, Caco2 | Apigenin | Cell-cycle arrest |
| HT29 | Dietary flavone | Induction of apoptosis |
| S/KS/FI | Butyrate | Induction of apoptosis |
| HT29, S/KS | NS398 | Induction of apoptosis |

BRINE SHRIMP LETHALITY BIOASSAY:

Many new natural compounds are isolated, characterized, and published without any biological testing whatsoever. Without accompanying biological data, the discovery of new medicinal plant constituents is nothing more than pure Phytochemistry. There is a real need for reliable, general bioassays which can detect a broad spectrum of pharmacologic activities in higher plants. Desiring a rapid, inexpensive, in-house, bioassay for screening and fractionation monitoring, of our physiologically active plant extracts, we have been using a tiny crustacean, brine shrimp, as the general bioassay tool. A general bioassay that appears capable of detecting a board spectrum of bioactivity present in crude extracts is the brine shrimp lethality bioassay (BSL). The technique is easily mastered, costs little, and utilizes small amount of test material.

Brine shrimp have been previously utilized in various other bioassay systems, namely, analysis of pesticidal residues, mycotoxins, stream pollutants, anesthetics, dinoflagellate toxins, Morphine like compounds, toxicity of oil dispersants, di-n-alkyl pthalates and co-carcinogenic phorbol esters etc. The assay is also widely being used for evaluating the cytotoxicity of plant based compounds. Most of workers have made use of the hatched napulii, although

inhibition of hatching of eggs has also been studied. B.N. Mayer et al developed "Brine shrimp Lethality Bio-assay" in 1982. The basic principle behind this method is "the toxicology is simply pharmacology at higher doses. Thus if we could be able to isolate toxic compounds, a lower non toxic dose might elicit a useful, pharmacological, perturbation on physiological system, especially cytotoxic compounds for the treatment of cancer. The main advantage of this bioassay is simple, (do require few apparatus), rapid (overall screening is finished in 72 hrs), inexpensive (does not require any special apparatus & does not require aseptic condition) and is an in house bioassay. The eggs of brine shrimp, "Artemia salina leach", are readily available at low costs at any pet shop as a food for tropical fish, and they remain viable for years in the dry state. Upon being placed in a brine solution, the eggs hatch within 48 hrs providing large number of larvae (nauplii). The organism is now suggested as a convenient probe for pharmacologic activities in plant extracts which may be manifested as toxicity towards the newly hatched napulii. For the study, plant extracts, fractions, or pure compounds are tested at initial concentrations of 10, 100 and 1000 ppm ($\mu\text{g/ml}$) after being added to vials or test tubes containing brine and ten shrimps in each of three replicates. Survivors are counted after 24 hrs,

and the percentage of deaths at each dose are recorded. These data can then be analyzed with Finney computer program to determine LC50 values.[14-17]

MTT-MICROCULTURE TETRAZOLIUM ASSAY:

In the MTT assay, the tetrazolium salt, 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), is actively absorbed into cells and reduced in a mitochondrial dependent reaction to yield a formazan product. The product accumulates within the cell since it cannot pass through the cell membrane. Upon

addition of DMSO, isopropanol, or other suitable solvent, the product is solubilized and liberated, and is readily quantified colorimetrically. The ability of the cells to reduce MTT provides an indication of mitochondrial integrity and activity, which is interpreted as a measure of cell viability. The assay is suitable for a variety of cell lines displaying exponential growth in culture and a relatively high level of mitochondrial activity. It should be noted that some known compounds selectively affect mitochondria, resulting in an overestimation of toxicity.[20,21]

Table 3: Other tetrazolium salts

| Tetrazolium salts | λ_{\max} (nm) | Solubility |
|---|-----------------------|-----------------|
| 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) | 550–600 nm | Water insoluble |
| 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide sodium salt (XTT) | 550–600 nm | Water soluble |
| 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt (WST-1) | 420–480 nm | Water soluble |

SULPHORHODAMINE B (SRB) ASSAY:

For the anticancer screen application, there were two especially troublesome problems encountered with the tetrazolium assays that eventually prompted the development of a third alternative microculture assay method. For either MTT or XTT, tetrazolium reduction was dependent on the cellular generation of NADH and NADPH. This raised concern about the influence of glucose concentration on the formation of the colored tetrazolium formazan, which was measured colorimetrically as an estimate of cellular growth or viability. Studies with MTT indicated that a progressive reduction in MTT specific activity (MTT formazan

formed/pg cell protein), which was observed during the course of a typical 7-d assay, was paralleled by a progressively decreasing glucose concentration. For XTT, there was a further problem resulting from the additional requirement of an electron transfer reagent, phenazine methylsulfate (PMS), to promote adequate cellular reduction of the tetrazolium. In an attempt to identify a suitable, nontetrazolium assay for use in the in vitro primary drug screen, a series of protein and biomass stains were investigated. These included anionic dyes that bound to the basic amino acid residues of proteins, as well as cationic dyes that bound to the negative, fixed charges of biological

macromolecules. Of all the reagents tested, sulforhodamine B (SRB) gave the best combination of stain intensity, signal-to-noise ratio, and linearity with cell number. SRB is a bright pink anionic dye that, in dilute acetic acid, binds electrostatically to the basic amino acids of TCA-fixed cells.[25]

TRYPAN BLUE EXCLUSION:

Measuring percentage Trypan Blue exclusion can rapidly assess cell viability and confirm whether cell culture conditions are optimal. Cells that are viable exclude Trypan Blue, while cells that have died are stained by this dye. Trypan Blue exclusion measurement is also useful for determining whether cells in culture have escaped breakage or disruption and to assess apoptosis. Trypan Blue exclusion measurement therefore has utility in assessing viability and also in assessing cell mortality in response to environmental insult. The staining procedure with this dye is simple. Determination of percentage Trypan Blue exclusion requires only a light microscope. Typically, a small percentage of cells are harvested for trypan Blue exclusion measurement. Determination of Trypan Blue exclusion is performed at one or several predetermined time points.[24,25]

CRYSTAL VIOLET INCLUSION:

Crystal violet inclusion measurement detects cell lysis. This dye stains viable cells that adhere to their culture vessel. Lysed cells simply fall away from their vessel surface and are not stained by this dye. Crystal violet is used in a standard bioassay for TNF- α . In this bioassay, fibroblasts are grown as a monolayer in the presence of actinomycin D. To these cells, increasing concentrations of TNF- α are added. Cells that are lysed in the presence of TNF- α are subsequently stained by crystal violet, whereas intact cells are stained by this dye. Crystal violet-stained cells are then detected by spectrophotometric measurement. This method has the limitation of

detecting only adherent cells and only permits measurement at a single predetermined time point. In other words, no continuous monitoring can be performed by this method.[27]

DISCUSSION

Comparison (Table 3) of these four autochthonous models shows differences in handling and biological behavior e.g., growth, invasion, and spread. In the DMH model induced tumors spread over the whole gut. Tumors also arose in other organs. This model allowed the hypotheses resulting from epidemiology or etiology regarding the whole bowel to be tested. In the three other models tumors were induced by rectal instillation. A high incidence of adenomas and carcinomas specifically in the large intestine were found. In addition, the site and time of tumor appearance was constant. Differences in the macroscopic appearance of the lesion - polypoid or ulcerative – allows the choice of a specific model for different clinical questions. The advantage of these models is the constant localization of tumor appearance (distal colon), allowing control of tumor development by colonoscopy. Also the cytostatic effectiveness of test substances in the colon can be evaluated within a short period by endoscopic observation of the tumor growth. For the form and rate of application and its results we prefer the AMMN model for our chemotherapy studies [5]. This tumor model which was developed by Schmfuhl and coworkers is today one of the best techniques for inducing autochthonous colon cancers. Table 2 contains different cell lines with their comparative test compounds used for study and the purpose of study e.g. inhibition of growth, induction of apoptosis and cell cycle arrest. Such cell lines can be easily used for high-throughput screening. On the other hand, they have been established from carcinomas or even metastases that have already accumulated many

genetic defects and genetic instabilities [10]
Table 3 shows the tetrazolium salts with their
lambda max and solubility in water.

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