THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

Establishment and characterization of new cell lines derived from chemically induced experimental tumors

by Gyorgy Trencsenyi

Supervisors:

Gaspar Banfalvi, Ph.D.
Janos Hunyadi, M.D., Ph.D.

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF CLINICAL MEDICINE

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Supervisors:
Gaspar Banfalvi, Ph.D.
Janos Hunyadi, M.D., Ph.D.

Head of the Examination Committee:
Zoltan Nemes, M.D., Ph.D.

Members of the Examination Committee:
Sara Toth, Ph.D.
Peter Kovacs, Ph.D.

The Examination takes place at
Department of Pathology
Medical and Health Science Center, University of Debrecen,
at 11:00 on March 4, 2010

Head of the Defense Committee:
Zoltan Nemes, M.D., Ph.D.

Reviewers:
Zoltan Szentirmay, M.D., Ph.D.
Sandor Sipka, M.D., Ph.D.

Members of the Defense Committee:
Sara Toth, Ph.D.
Peter Kovacs, Ph.D.

The Ph.D. Defense takes place at
The Lecture Hall of 1st Department of Medicine,
Institute for Internal Medicine, Medical and Health Science Center,
University of Debrecen, at 13:00 on March 4, 2010

INTRODUCTION

The attention in the last two decades has turned towards the chemoprevention of tumors and deseases of the heart and blood vessel system, which are responsible for 80% of the premature deaths.

The bibliography of the chemoprevention of tumors has become immense within the last few years, which indicates the intensity of the research in the field. Despite of the intensity of the research the chemo-prevention of tumors could not live up to expectations due to methodical difficulties. Testing a new molecule can theoretically not differ from the methods of preclinical and clinical pharmacology, but many problems arise in practice. Cell culture and molecular biology methods are not suitable, yet for measuring the preventive effects. and the formation of metastasis, therefore tumor-bearing animals are required. It is not clear, which would be the most reliable way ~~is~~ to induce tumor in experimental animals: the use of chemical carcinogens, virus or ionizing radiation? It is a question reasonable to ask, as chemoprevention seems to be a promising approach, nevertheless still uncertain, whether the effect can be generalized, is it specific for the chemical carcinogen or for the animal virus, by which the tumor was induced. It is also debated, whether the developing tumor becomes a carcinoma, a sarcoma or a leukemia, and which type of tumor should be used to test the effect of the molecule. It is not certain either, whether the tumor induced this way would give metastasis, although the lethal outcome of human tumors would indicate to the formation of metastasis. A further problem is that neither carcinogens, nor viruses, nor radiations provide a 100% carcinogenic? effect, and a relatively large number of control and treated animals is required, to clarify the carcinogenic effect and their treatment may take several months or even 1-2 years. These experiments generate heated protest against animal experiments, besides being time and cost consuming. After accomplishing all these animal experiments human tests may follow, which unlike the clinical pharmacological examinations require unusually large number of voluntary control and treated groups. Moreover, the evaluation of results that follow may take years or rather decades. These methodical hindrances make the research of chemo-prevention difficult. The experiments we have made recently, intended to simplify the preclinical examinations and to make them more reliable. Our aim was to develop a method, that a) gives reliable results, b) scrifices a relatively small number of animals, c) gives results within a short period of time, d) takes into consideration all three tumor types and e) is able to following closely the spread of metastasis. Based on examinations

our experimental system turned out to be suitable achive these goals. Our experimental system included the implantion of 10^6 hepatocarcinoma, mesoblastic nephroma or myelomocytic leukemia cells under the renal capsule of rats. These tumor cells were obtained from tumors induced in rat by chemical carcinogens and were then converted to tumor cell lines which provided us with reliable means for *in vivo* tumor formation.

Chemical carcinogens

In the early 20th century, the etiology of cancer was thought to be either; 1) viral, 2) chemical or 3) genetic. Although, each school often predicted that "their mechanism" would be universal, each of these ideas has been proven to be correct. We now know that each factor can be important to human cancer incidence. The history of a chemical etiology for cancer began with work at the end of the 18th and beginning of the 19th century Europe where physicians had noted correlations between nasal and mouth cancer and a high incidence of scrotal cancer in chimney sweeps. The story of the English physician Percival Pott is often recounted as a primary event in our understanding of chemical carcinogenesis. In addition, his observation led to an early scientific focus on the mechanism of action of "coal tar" carcinogens. The early coal tar connection led to the first chemical carcinogenesis experiment by Yamagiwa and Ichikawa who showed that rabbit ears painted with coal tar developed skin tumors. This observation led to the isolation and structural elucidation of the first carcinogens, dibenzanthracene and benzo(a)pyrene by Kennaway and Cook between the 1930s and 1950s. The first chemically identified carcinogens were the polycyclic aromatic hydrocarbons. They are composed of variable numbers of fused benzene rings that form from incomplete combustion of fossil fuels and vegetable matter, and they are common environmental contaminants. The polycyclic aromatic hydrocarbons are chemically inert and require metabolism to exert their biologic effects. This is a multi-step process, involving initial epoxidation (cytochrome P-450, encoded by the CYP1A1 gene), hydration of the epoxide (epoxide hydrolase), and subsequent epoxidation across the remaining olefinic bond (principally CYP3A4). The result is the formation of the ultimate carcinogenic metabolite a diol-epoxide. Aromatic amines are another class of chemical carcinogens, and 4- aminobiphenyl is thought to be responsible for bladder cancer among workers in the rubber industry. This and many related compounds are components of cigarette smoke, diesel exhaust, and the pyrolysis of certain foods. Aflatoxins are metabolites of

Aspergillus flavus. These fungal mutagens contaminate improperly stored cereals, grains, and nuts. A positive correlation exists between dietary aflatoxin exposure and incidence of liver cancer in the developing countries, where grain spoilage is high. Carcinogenic *N*-nitrosamines are ubiquitous environmental contaminants and can be found in food, alcoholic beverages, cosmetics, cutting oils, hydraulic fluid, rubber, and tobacco. *N*-nitrosodimethylamine undergoes *a*-hydroxylation to form an unstable *a*-hydroxynitrosamine. The breakdown products are formaldehyde and methyl diazohydroxide. The alkyl groups of compounds, such as methyl diazohydroxide, are good leaving groups and, thus, are powerful methylating agents that can add a small functional group (small alkyl adduct as opposed to the bulky aryl adducts formed by the carcinogens discussed earlier) at more than 10 different sites in DNA.

Multi-stage carcinogenesis

Carcinogenesis can be divided conceptually into four steps: tumor initiation, tumor promotion, malignant conversion, and tumor progression. Tumor initiation results from irreversible genetic damage. For mutations to accumulate, they must arise in cells that proliferate and survive the lifetime of the organism. A chemical carcinogen causes a genetic error by modification of the molecular structure of DNA that can lead to a mutation during DNA synthesis. Most often, this is brought about by formation of an adduct between the chemical carcinogen or one of its functional groups and a nucleotide in DNA. The carcinogen–DNA adduct formation is central to theories of chemical carcinogenesis, and it can be considered to be a necessary, but not a sufficient, prerequisite for tumor initiation. DNA adduct formation that results in either the activation of a proto-oncogene or the inactivation of a tumor suppressor gene can be considered to be a tumor initiating event. Tumor promotion comprises the selective clonal expansion of initiated cells. Because the accumulation rate of mutations is proportional to the rate of cell division, or at least the rate at which stem cells are replaced, it follows that clonal expansion of initiated cells produces a larger population of cells that are at risk of further genetic changes and malignant conversion. Malignant conversion is the transformation of a preneoplastic cell into one that expresses the malignant phenotype. This process requires further genetic changes. Tumor progression comprises the expression of the malignant phenotype and the tendency of already malignant cells to acquire more aggressive characteristics with time. Metastasis also may involve the ability of tumor cells to secrete proteases that allow invasion

beyond the immediate location of the primary tumor. A prominent characteristic of the malignant phenotype is the propensity for genomic instability and uncontrolled growth. There are several ways in which the chemical structure of DNA can be altered by a carcinogen, including the formation of bulky aromatic-type adducts, alkylation (generally small adducts), oxidation, dimerization, and deamination. Chemical carcinogens also can cause epigenetic changes, such as alteration in DNA methylation status, that can lead to silencing of specific gene expression.

Transplantation of experimental tumors

At the end of the XIX. century more and more scientists found the tracking of spontaneous tumor developement in human and in experimental animals insufficient, they tried to transplant spontaneous tumors of experimental animals in order to get copious amount of tumorous material suitable for observation. Vets were the first who tried to transplant tumors of domestic animals into animals of the same species, with moderate success. In 1889 Hanau found a rat tumor, that he vaccinated succesfully into other rats. Five years later Moreau found a spontaneous mouse-tumor, that he inoculated succesfully into other mice. In the first years of the XX. Century Jensen, Bashford and Murray found that the inoculated tumors act in every respect like human malignant tumors, and they develop from the cells of the inoculated tumor instead of those of the host animal, which refer to a proper transplantation. The year 1934 brought a further change: Andervont showed that not only the spontaneous tumors can be transplanted, but also the chemically produced experimental tumors. During the transplantations it was observed, that the tumor strains cannot be transplanted into species different from the donor animal, and problems may occur even within the same species. E.g. Michaelis could not transplant the Jensen-type mouse tumor from mice bred in Coppenhagen into mice bred in Berlin. This perception led Little to produce the first inbred mouse-strain consisting of genetically equivalent individuals, by which he opened a new chapter in the field of cancer research as well as in the transplantation immunology. From further experiments it turned out, that breaking through the transplantation resistence any human or animal tumor can be succesfully transplanted into nude mice. Bogden et al. took this observation as their starting point when setting human tumor pieces under the renal capsule of nude mice in order to test the efficiency of chemotherapy drugs against human tumors.

Metastatic models

Metastasis, the spread of a tumor from its primary site to other parts of the body, continues to be the most significant problem in the field of cancer. Patients with metastatic disease or those who develop metastases after the treatment of the primary tumor carry an universally grave prognosis. To improve the outcome of the treatment for these patients a broader understanding of the biology of metastases is necessary. The biological complexity that characterizes metastasis requires complex experimental systems for its study. To a large extent the modeling of this biological complexity is only possible using animal models. Such transplantable models can be divided into two broad groups, syngenic models and xenograft models. Syngenic transplantable models most often refer to mouse or rat (murine) cancer cell lines or tissues that result in tumors in inbred animals of the same genetic background as the derived cell line or tissue. Until recently syngeneic cell lines have either been derived from carcinogen-induced tumors or tumors that spontaneously develop in a particular mouse or rat. The advantage of syngeneic models is that the transplanted tissues, the tumor microenvironment, and the host are from the same species. This is particularly important when considering the close interaction between tumor and host characterized by the process of metastasis. However, these model systems lack many of the important features of human tumors.

Human-mouse xenograft models define the other major category of transplantable cancer models used to study metastasis. Such xenograft models refer to human cancer cell lines or tissues that can be transplanted into immunocompromised animals and effectively grow tumors. The resultant tumors that emerge from xenograft transplantations are a mosaic of human cancer cells and murine stromal cells. Several lines of evidence suggest the importance of cancer cell--stromal cell interactions in the biology of cancer progression and metastasis. For some pathways, species specificity does not allow this interaction to occur across species boundaries. The impact of this more limited tumor-stromal interaction must be considered in the use of xenograft models.

The most common site of tumor cell injection employed for experimental metastasis models is the circulatory system. Experimental metastasis refers to the injection of tumor cells directly to the systemic circulation. Depending on the site of injection and tropism of the tumor cell, distant metastases may (or may not) develop at a number of anatomic locations throughout the body. The site of injection largely defines the site to which metastases develop in these experimental systems. Tail vein injection results primarily in pulmonary metastases. In contrast, intrasplenic or

portal vein injection of tumor cells is the most common site employed for developing metastasis in the liver. Intracardiac injection of cells may result in metastases to several sites, including bone. These tumor-host determinants have been described in the seed and soil hypothesis, first described by Paget. This hypothesis suggests that the eventual outgrowth of a tumor, in this case at a metastatic site, is defined by determinants of the tumor cell (seed) and the ability of the tumor cell to receive appropriate growth and survival signals from its microenvironment (soil).

Historically, transplantable tumor models were characterized by and selected for rapid primary tumor growth at subcutaneous (s.c.) (heterotopic) sites. In this setting, it was uncommon to observe spontaneous metastasis to distant sites. As such transplantable models were often labeled as 'non-metastatic'. Application of the 'seed and soil' hypothesis to transplantation modeling resulted in the use of orthotopic transplantation of tumor cells into mice. Orthotopic transplantation refers to the delivery of cancer cells to the anatomic location or tissue from which a tumor was derived. The use of orthotopic injection transplantation has resulted in tumor models that may more closely resemble human cancers including tumor histology, vascularity, gene expression, responsiveness to chemotherapy and metastatic biology. As more has been learned about the importance of host-microenvironment interactions it is understandable why orthotopic tumors are preferred over more conventional flank implantation (s.c.) models. That orthotopic models are more frequently associated with metastasis than s.c. tumor injection of cells, lends support to the value of providing more relevant host--tumor interactions. Similarly, the fact that spontaneous metastases arise from a primary transplanted tumor provides an opportunity to study the metastatic process and many aspects of the metastatic cascade that are bypassed using experimental metastasis models. Orthotopic transplantation of cancer cells may come from direct injection of tumor cells or the surgical implantation of intact fragments of a tumor. For many orthotopic models the use of surgical implantation of fragments improves the reproducibility and metastatic outcome within the model.

AIMS

As satated in the Introduction, we wished to develop a method to study tumor chemoprevention and to obtain reliable result on relatively few animals within a short period of time, by using three types of tumor (carcinoma, sarcoma, leukaemia) and following the spread of

tumor cells to metastatic sites. My work focused primarily on the biological characterisation of different types of tumors. This was done achieving the following aims:

1. We have transplanted conventionally tumor pieces of chemically induced, rat-originated hepatocellular carcinoma (He/De), mesoblastic nephroma (Ne/De), and myelomonocytic leukemia (Myl/De) tumors, which however make the determination of the quantity of cells in the implantatum impossible. Our aim was to prove that chemically induced tumors can be grown *in vitro*, and that cell lines can be established from this cell culture. These cell lines are sustainable, can be kept frozen for an unlimited time and reimplanted any time under the renal capsule.

2. Our second aim was to confirm that the chemically induced leukemia tumor cells (My1/De), implanted under the renal capsule, generate invariably lymph metastasis. It was our goal to determine the time of metastatic spread after implanting 10^6 cells.

3. The third aim was to follow the dispersion of chemically induced tumor-cells with diagnostic imaging methods. This was done by measuring the uptake of ^{18}FDG in all the three tumor-types formed after the implantation of tumor cell lines originating from chemically induced tumors. We compared the mechanism of the ^{18}FDG uptake in the three tumor-types and in tumor-free tissues. It was our intention to prove, that the tissue distribution of ^{18}FDG used in rats may be used in human diagnostic practice.

4. Aim 4 was to compare the chromatin-structure and chromatin-condensation processes of chemically induced hepatocarcinoma tumor-cells and that of normal and regenerating rat liver cells.

MATERIALS AND METHODS

Animals

The experiments were carried out using male and female inbred Lomg-Evans and Fischer 344 rats. Animals were kept in a conventional laboratory environment and fed on a semi-synthetic diet (Charles River Mo, Kft, Godollo, Hungary) and tap water ad libitum. Animals received humane care according to the criteria outlined in the UK “Guide for the Care and Use of

Laboratory Animals", authorized by the Ethical Committee for Animal Research, University of Debrecen.

Experimental tumors

Three types of tumors were used in this study: epithelial liver carcinoma (He/De), mesenchymal mesoblastic nephroma (Ne/De) and myelomonoblastic leukemia (My1/De). He/De and Ne/De were isolated from Fischer 344 rats after the treatment of one-day-old infant rats with 125 μg/animal i.p. injection of nitrosodimethylamine in saline. My1/De was isolated from Long-Evans rats treated in 50-day-old animals 3 times by intravenous injection of 8 mg/animal 7,12-dimethylbenzanthracene. Tumors (He/De, Ne/De), spleen (My1/De) were removed 5-7 months after chemical tumorigenesis, minced into smaller pieces and frozen in liquide nitrogen.

Experimental surgery

The aim of the surgical operations was to place GelasponR discs or lymph nodes under the capsule of the left kidney. To transplant cells, gelatin sponge discs ~~of~~ 4 mm in diameter and 1 mm thickness were cut from Gelaspon^R sponge (Germed, Rudolstadt, Germany) and sterilized. To transplant tumor cells, 10^6 He/De, Ne/De or My1/De cells in 10 μl saline were placed onto the Gelaspon^R disc. The implantation of parathymic lymph nodes (PLNs) was carried out in the following way: the left parathymic lymph nodes were excised from 12 tumor-free (control) and from 12 tumor-bearing rats and the surrounding fat was removed. In the case of India ink, 10 μl Pelikan ink (Gunther Wagner, Pelikan Werke, Hannover, Germany) was placed onto the gelatin disc. Animals were euthanized 3, 6, 24 and 48 h after implantation (2 animals, each) and tissue sections were prepared from the kidney and from the parathymic lymph nodes. The next series of experiments aimed to define the time of appearance of metastases. In the first series of experiments related to the temporal aspects of tumor metastasis 10^6 He/De and Ne/De cells were implanted in six F344 male and six F344 female rats. Animals were euthanized 1, 3 and 6 days (2 animals each) after implantation, their parathymic lymph nodes were removed and these glands were implanted under the renal capsule of other six (3x2) tumor-free male rats. These experiments were based on the assumption that if the lymph nodes contained metastatic cells, then their implantation would cause tumor growth within two weeks similary to the direct implantation of He/De or Ne/De cells. Another series of experiments were modified in such a

way that after 1, 3 and 6 days of tumor cell implantation, not the parathymic lymph nodes, but the left kidney carrying the implanted tumor cells was removed. Nephrectomy was followed by a further 13, 11 and 8 days of observation, respectively. After two weeks (1+13, 3+11, 6+8 days) the parathymic lymph nodes were removed and He/De cell containing lymph nodes were implanted under the renal capsule of tumor-free male rats, and Ne/De cell-containing lymph nodes in female rats. Tumor growth was measured 2 weeks after parathymic lymph node implantation. In every case experimental animals were anesthetized by i.p. administration of 3 mg/100g pentobarbital (Nembutal). The retroperitoneum was opened by laparotomy, the kidney was pulled out, and either India ink, tumor cell- or lymph node containing Gelaspon disc, was placed under the renal capsule and the kidney was placed back in the abdominal cavity. Stitches were put in the wound and autopsy and autoradiographic experiments were carried out two weeks later unless otherwise noted.

Establishment of tumor cell lines

In case of He/De and Ne/De, tumor pieces (freshly isolated or frozen) were further minced into 2×2×2 mm pieces and incubated for 3 h at 37˚C in RPMI-1640 medium containing 100 mg collagenase I, 10 mg hyaluronidase and 30 µl DNase I per 100 ml. After digestion, the mixture was filtered through four layers of sterile gauze, washed and resuspended in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. After overnight incubation at 37˚C in a 5% carbon dioxide (CO_2) atmosphere, nonadherent cells were discarded and adherent cells were subcultured. The primary cell culture was continuously grown and after subculturing for a further 20 days the new cell lines were established, frozen in liquid nitrogen and used for further experiments. Both cell lines (He/De and Ne/De) were used as exponentially growing monolayer cultures (37˚C, 5% CO_2), maintained by daily passage in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. In case of My1/De, the *femur* of Long-Evans rats was aseptically removed, the bone marrow was flushed out of the bone with a syringe filled with saline and the bone marrow was resuspended is saline. The non-adherent leukemic cells were grown in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Isolation of F344 rat fibroblast cells

As control, freshly isolated rat fibroblast cells were used. A pregnant rat (7 days of pregnacy) was anesthetized, embryos were removed and placed in sterile PBS. Embryos were minced into small (≈2x2 mm) pieces and digested for 3h at 37ºC in Hank's solution containing 0.25% trypsin. The cell suspension was filtered through four layers of sterile gauze, washed, resuspended in RPMI 1640 medium supplemented with 10% FBS and antibiotics. After overnight incubation at 37ºC in a 5% carbon dioxide atmosphere, the nonadherent cells were discarded and adherent cells were used as control.

Isolation and primary culture of nonproliferating F-344 rat hepatocytes

Animals (F-344 rats of 250 g in weight) were anesthetized by i.p. administration of 3 mg/100g pentobarbital (Nembutal), then we opened the abdominal cavity. The portal vein was cannulated by a 21-ga butterfly needle. The *arteria hepatica* was strangulated. After opening the chest cavity, the vena cava inferior was cut-off and the perfusion pump was activated (rate of flow: 25 ml/min) to force the perfusion medium (HBSS) containing 2 U per ml heparin through the liver. After 10 minutes, the medium was changed to perfusion medium containing collagenase (20 U collagenase type 1 in HBSS without heparin). After a 15-min perfusion with collagenase medium, the lobes of the liver were removed, cut into 2 mm pieces, then placed into 40 ml perfusion medium at 4 ^{o}C. After shaking, the stomal tissue was removed by successive filtrations through 250- and 62 µm filters. The mixture was washed and the cells were resuspended in Ham's F-12 medium supplemented with 17% FBS and antibiotics. After overnight incubation at 37ºC in a 5% carbon dioxide atmosphere, the nonadherent cells were discarded and adherent cells were used.

Isolation of regenerating hepatocytes

For partial hepatectomy (liver lobectomy) F-344 rats (250 g) were used. The rats were fasted preoperatively for 24 hours. After pentobarbital anesthesia, the abdomen from the lower part of the sternum (xiphoid process) was shaved and sterilized. The abdomen was then opened by means of a longitudinal, mid-line incision from the xiphoid process caudally to about 3 to 4 cm. The desired left lateral or left lateral and median lobes of the liver became readily accessible. These lobes are attached to separate pedicles to which a hemostat was connected. A silk ligature

was firmly tied around one or both pedicles beyond the hemostat, depending on whether the left lateral lobe alone (in most of our experiments) or the left lateral and median lobes were to be removed. Stitches were put in the wound. For the 1st postoperative day the animals were kept in a warm room (temperature about 27 ^{o}C). The isolation of the regenerating cells from the liver was carried out four days after the hepatectomy by the same method what we used for the isolation of resting hepatocytes.

In vitro uptake of ^{18}FDG glucose analogue

Exponentially growing, daily subcultured He/De, Ne/De and My1/De cells were used for the *in vitro* uptake of glucose analogue. Cells of the hepatocellular He/De cell line, mesenchymal Ne/De cell line and the myelomonocytic leukemia My1/De cells were washed and resuspended in PBS. Freshly isolated fibroblasts and bone marriow suspension served as control. Samples (10^6/ml) were preincubated at 36 ^{o}C for 10 minutes in PBS containing 1 mM α-D-glucose. Preincubation was followed by the addition of 185 kBq ^{18}FDG to each sample. After the addition of the radiotracer, the cells were incubated at 36 ^{o}C for 15, 30 and 60 minutes and the uptake was terminated by the addition of 10 ml ice-cold PBS. Cells were washed three times with cold PBS, resuspended in 1 ml cold PBS and the radioactivity was measured. In a separate experiment the uptake of ^{18}FDG was measured in the presence of 5 μM cytochalasin B, and the cells incubated for 60 min. The uptake of ^{18}FDG was expressed as percentage of the total radioactivity of ^{18}FDG added to the cells.

Whole-body autoradiography

On day 14 after implantation, ~~12~~ control and tumor-bearing rats were anesthetized and the radioligand 18FDG (fluoro-deoxyglucose; 15.0 MBq in 1 ml saline) was injected into the left femoral vein of each rat. Animals were euthanized 60 min after the administration of 18FDG with 300 mg/kg pentobarbital. Each animal was then embedded in an ice-cold 1% carboxymethylcellulose solution. After being frozen in liquid nitrogen, 60 μm thick cryostate sections (Leica CM 3600 cryomacrotome; Nussloch, Germany) were cut in the sagittal plane. Sections were exposed to phosphor imaging plates (GE Healthcare, Piscataway, NJ, USA). For anatomical correspondence, true-color images of the same sections were also obtained by a transparency scanner (Epson Perfection 1640; EPSON Deutschland GmbH, Meerbusch,

Germany). Autoradiography and transmission images were overlaid to enable merging of the functional and anatomical information. Phosphor image analysis used the average of 16 sections calculated by using the Image Quant 5.0™ (GE Healthcare, Molecular Dynamics) image-analysing software. Results were expressed in intesity/pixel units.

Organ distribution of ^{18}FDG glucose analogue

In experiments where the organ distribution was investigated, six male and six female F344 rats after 14 days of subcapsular transplantation of 10^6 Ne/De or He/De cells, were anaesthetized with pentobarbital and similarly to the previous series of experiments animals were given i.v. 15.0 MBq 18FDG in saline. After 1 h blood samples were taken from the aorta. The tumor, the kidney, the thymus, the abdominal rectal muscle and the parathymic lymph nodes were removed. Three tissue samples were taken from each organ and their activites were measured with a gamma counter (Canberra Packard). The weight and the radioactivity of the samples were used to determine the differential absorption ratio (DAR). DAR was calculated as:

$$DAR = \frac{\text{(accumulated radioactivity/g tissue)}}{\text{(total injected radioactivity/body weight)}}$$

Immunocytochemistry, immunohistochemistry

For ~~the~~ immunocytochemistry, hepatocarcinoma (He/De) and mesenchymal nephroma (Ne/De) cells were cultured on the surface of 20x20 mm rectangular coverglasses (Spectrum 3D, Debrecen). Cells were fixed in 4% paraformaldehyde for 15 min, and then washed in PBS. For immunohistochemistry, the paraffin embedded sections of the tumors were deparaffinized, rehydrated and then washed in PBS. Non-specific binding sites were blocked with PBS containing 1% bovine serum albumin at 37 ^{o}C for 30 min. After washing with PBS cells were incubated with anti-GLUT-1, anti-GLUT-3 and anti-TGF-β1 antibodies at 4 °C overnight. A 250-fold dilution of primary antibodies was applied. To visualize the primary antibodies Texas red conjugated secondary antibody was used at 1:1000 dilution.

Western blot analysis

Total cell lysates were examined by Western blot analysis. Samples for SDS-PAGE were prepared by the addition of 100 μL of fivefold concentrated electrophoresis sample buffer (20

mM Tris-HCl pH 7.4, 0.01 % bromophenol blue dissolved in 10 % SDS, 100 mM ß-mercapthoethanol) to cell lysates and boiled for 10 min. About 60 µg of protein was separated by 7.5 % SDS-PAGE gel for detection of GLUT-1, GLUT-3 and TGF-β1. Proteins were transferred electrophoretically to nitrocellulose membranes. After blocking in 5 % non-fat dry milk in PBST (phosphate buffered saline with 0.1 % Tween 20, 20 mM Na_2HPO_4, 115 mM NaCl; pH 7.4), membranes were washed and exposed to the primary antibodies overnight at 4 °C. Polyclonal anti-GLUT1 antibody (Abcam Inc., Cambridge, MA, USA) in 1:250 dilution, polyclonal anti-GLUT3, TGF-β1 (Abcam Inc., Cambridge, MA, USA) in 1:400 dilution were used. After washing for 30 min with PBST membranes were incubated with secondary antibodies, anti-rabbit IgG (Bio-Rad Laboratories, CA, USA) in 1:1000 dilution in PBS containing 1 % non-fat dry milk for 1 hr at room temperature. Signals were detected by enhanced chemiluminescence (Pierce, Rockford, IL., USA) according to the instructions of the manufacturer.

Reversible permeabilization

This method enables the cell membrane to be permeabilized and resealed while maintaining viability during the cell cycle. Briefly, 1 mL of hypotonic buffer was added to 5 x 10^7 cells in the presence of Dextran T-150 as a molecular coat to prevent cells from disruption. Permeabilization lasted for 2 min at 0 ^oC. For reversal of permeabilization, the hypotonic solution was replaced by 10 ml growth medium containing 10% FBS. Cells were sealed after permeabilization by incubation in a carbon dioxide (CO_2) incubator at 37 ^oC and 5% CO_2 for 3 h to avoid their stickiness, but allowing them to open anytime during the cell cycle.

Isolation of nuclei

Based on the cyclic character of chromatin unfolding and chromosome condensation, isolated cell poulations were treated with colcemid to arrest the cycle in metaphase. Cells (10^6) were resuspended in growth medium after reversal of permeabilization and treated with 0.1 µg/ml colcemid for 2 h at 37 ^oC under 5% CO_2. Cells were detached with trypsin, washed with PBS and incubated at 37 ^oC for 10 min in swelling buffer, followed by centrifugation at 500×*g* for 5 min. Nuclei were isolated by the slow addition of 20 volumes of fixative solution (methanol:glacial acetic acid, 3:1).

Spreads of nuclear structures

Preparation of nuclei for spreads of chromatin structures used the method developed for metaphase chromosomes. Nuclei were centrifuged at 500×*g* for 5 min, washed twice in fixative and resuspended in 1 ml fixative. Nuclei were spread over glass slides dropwise from a height of approximately 30 cm. Slides were air dried, stored at room temperature overnight, rinsed with PBS and dehydrated using increasing concentrations of ethanol.

Visualization of chromatin structures and chromatin image analysis

Dehydrated slides containing isolated chromatin structures were mounted in 35 µl Antifade Medium under 24×50 mm coverslips. Blue fluorescence of DAPI was monitored with an Olympus AX70 fluorescence microscope.

The RGB (red-green-blue) images of nuclei were converted to grayscale intensity masks, using 50-50% information of the green and blue channels. To improve the comparability of images, the information range of images was normalized to 256-tonality scale. Pixel counts for each intensity value were plotted in the histograms. The entropy of the image was calculated from the upper half of the intensity range, the subtraction of which automatically removed background fluorescence. Irrespective of its size, the area and the contour of each chromatin image was calculated. The contour values were numerically divided by the area of the chromatin image in each nucleus. The resulting contour/area ratio shows the intensity-independent fragmentation of chromatin.

Statistical analysis

Statistical analysis was performed with Student's t-test. Results are expressed as mean±standard deviation (SD), $p<0.01$ was considered as significant.

RESULTS

Hepatocellular carcinoma (He/De)

Establishment of cell line from hepatocellular carcinoma and reimplantation measurements

Pieces from the chemically induced hepatocellular carcinoma were digested with proteolytic enzymes, then the filtered and washed single cell suspension was placed into culture vessels at at a density of 3×10^6. The doubling time of the adherent cells is 24 h. The absence of

the contact inhibition and the aggressive growth rate are typical features of these cells. The morphology and the ability of tumor and metastasis formation of the cells did not change after the 100th passage. The cultured hepatocellular carcinoma cells are small (7-9 μm in diameter), granular when stained with Giemsa or hematoxylin and eosin. At the early, preconfluent stage of low cell density in each serial subculture, it was possible to define contracted bipolar, polygonal, elongate fusiform and stellate cells, which are randomly distributed (no islands). Gelatin disc containing He/De tumor cells (10^6) were implanted under the left renal capsule. After 7 days of tumor cell implantation, the tumor infiltrated into the kidney causing its destruction.

Chromatin structures in resting, regenerating hepatocytes and in hepatocellular carcinoma cells (He/De)

Decondensed chromatin structures in resting hepatocytes

The analysis of chromatin structures by fluorescent microscopy revealed that nuclei of resting cells being in Go phase tended to maintain their round shape even if the nuclear membrane was disrupted. The chromatin in resting hepatocytes was in a highly decondensed state. The chromatin in the fixed nuclei appeared as a fuzzy, veil-like structure. The chromatin veil turned to loose chromatin ribbons. The nuclear material either maintained its round shape or the chromatin began to unfold to elongated supercoiled structures. Typical unfolded structures contained six chromatin clusters. The chromatin loops consisting of chromatin fibers, where the chromatin is seen as a continuous supercoiled structure without distinguishable chromosomes.

Chromatin structures of regenerating hepatocytes

Regenerating hepatocytes represented a fast growing cell population. The intermediates of chromatin condensation in this cell population represent structures characteristic to exponentially growing cells where most of the cells are in S phase. In this cell population further evidence for a continuous chromatin structure was provided by the examination of chromatin structures. We have seen the whole spectrum of intermediates of chromatin condensation. These structures included decondensed veil-like chromatin, polarized condensation inside the chromatin veil, which turned to supercoiled chromatin ribbon. Supercoiling led to the formation of chromatin

bodies. Continued supercoiling turned then the fibrous chromatin structure to thicker strings. In some cases early forms of chromosomes could be distinguishable.

Chromatin structures of hepatocellular tumor cells

The most characteristic chromatin structures isolated from these tumor cells were supercoiled ribbons and fibrous structures. The nuclei of these cells opened readily at the decondensed stage, making the fine chromatin veil visible as a faint halo. Another characteristic feature of chromatin from tumor cells is the high level of supercoiling observed throughout the process of chromatin condensation. The third, and probably the most important change in chromatin condensation relative to resting and regenerating cells was the formation of apoptotic bodies.

In vitro uptake of radiotracer glucose analogue in He/De cells

Exponentially growing, daily subcultured cells were used for the in vitro uptake of glucose analogue. The incorporation of ^{18}FDG is approximately 4-times higher in hepatocarcinoma cells than in control fibroblast cells. ^{18}FDG uptake indicates an aggressive growth rate and fast metabolism of HeDe cell line. Cytochalasin B is a known inhibitor of glucose uptake in cells. Cytochalasin B was applied at a concentration of 5 μM to demonstrate that ^{18}FDG uptake can be suppressed not only in the primary fibroblast cells serving as a control, but also in He/De tumor cells. As expected, this experiment proved that cytochalasin B inhibits glucose uptake in all cell types. The ^{18}FDG incorporation was 11-times less in the presence than in the absence of this fungal metabolite.

Detection of GLUT transporters in He/De cell line by immunocytochemistry and Western blot analysis

The expression of GLUT-1 and GLUT-3 glucose transporters was tested by immunocytochemistry and Western blot analysis. Results using this triple labeling procedure revealed high relative levels of the F-actin in fibroblasts. GLUT-1 expression was relatively low compared to the tumor cells, localized mainly to the lamellopodia, with low diffuse signals in the cytoplasm stored in the low density microsomes. GLUT-3 was almost undectable in fibroblasts. High levels of GLUT-1 and GLUT-3 expressions were seen in He/De cells, but among these, the GLUT-1

was the most widely expressed. This result was also supported by the high FDG uptake and by Western blot analysis. The GLUT-1 transporter is localized not only within the cell membrane but is also present in a diffuse form in the cytoplasm among the transporter proteins. We examined the levels of GLUT-1 and GLUT-3 in cell lysates prepared from fibroblasts and from the hepatocarcinoma tumor cell line using Western blot analysis. GLUT-1 is the most widely expressed isoform of mammalian glucose transporters that provides cells with their basic glucose requirement. The expression of GLUT-3 was low in fibroblast cells and in the He/De cell line.

Detection of GLUT transporters in He/De tumor by immunohistochemistry and Western blot analysis

Chemically induced hepatocellular tumors have not been previously tested for GLUT transporters. The visualization of GLUT-1, GLUT-3 transporters and TGF-β1 expression was facilitated by the use of antibodies against these proteins. Results obtained with this triple labeling procedure revealed low levels of GLUT-1, GLUT-3 and TGF-β1 in the tissue sections of kidneys obtained from tumor-free rats. In the hepatocarcinoma tumor GLUT-1 and TGF-β1 expression was high relative to the low GLUT-3 expression. These results were also supported by ^{18}FDG uptake and by Western blot analysis.

Transport of abdominal ink particles to thoracic parathymic lymph nodes

In the initial experiments subcapsular renal implantation of Gelaspon^{R} disc containing 10 μl India ink was carried out in 8 rats. After 24 h, ink particles occupied large areas of parathymic lymph nodes infiltrated by neutrophils and other inflammatory cells as well. The most pronounced discoloration of lymph nodes seen under the microscope 24 h after implantation with several recognizable ink granules. The transport of India ink particles from the renal capsule to parathymic lymph nodes could be perceived and examined with unaided eye. In the control experiment, where saline without ink was administered, normal parathymic lymph nodes were isolated. Twenty four hours after of India ink implantation, the parathymic lymph nodes were packed with the ink. Control kidney did not contain ink, but a significant amount of ink remained under the renal capsule after implantation.

Metastatic potential of He/De cells followed by whole body autoradiography, phosphor image analysis and tissue distribution of ^{18}FDG

The *in vivo* distribution of ^{18}FDG was studied in tissues isolated from tumor-bearing F344 rats. After the administration of the radioligand (15.0 MBq ^{18}FDG in saline) the animals were euthanized, dissected and the differential absorption ratio (DAR) was determined in blood plasma, muscle, HeDe tumor, liver, parathymic lymph node, thymus, and kidney. The biodistribution of ^{18}FDG in tumor-bearing rats was lowest in the resting muscle. The high levels of ^{18}FDG in the living part of hepatocarcinoma (He/De) tumor and in the parathymic lymph node reflected high *in vivo* proliferative activities. The radiotracer uptake was followed by whole body autoradiography. The ^{18}FDG distribution in tumor-bearing rats showed high local concentration in the the kidney indicating its rapid clearance. The rapid metabolic rates of He/De tumors are clearly visible in the primary tumors developed under the kidney. It is to be noted that the high ^{18}FDG concentration inside the kidney was found in the renal pelvis where the radiotracer is excreted and accumulates before being removed via the urine. Anatomical images serve to provide a view of selected regions of interest (ROI). The program gives the results in pixels of the selected ROIs, and the average pixel density of the selected area. The number of pixels corresponds to the radioactivity of the selected regions of interest. As a unit of pixel density (radioactive counts/pixel) the resting muscle of the hind leg of the euthanized, tumor-bearing rat was chosen. By taking the pixel intensity of resting striated muscle as one unit, the relative pixel densities were, in decreasing order: 14.23±2.6 in He/De tumor, 10.82±2 in parathymic lymph nodes, 2.35±0.2 in blood and 1.57±0.4 in liver.

Appearance of metastases

Next series of experiments aimed to define the time of appearance of metastases. We found that parathymic lymph nodes isolated from tumor-bearing animals after one and three days of tumor cell implantation did not cause tumor formation upon implantation of these isolated lymph nodes to other rats. To the contrary, six days after He/De cell implantation, the isolated parathymic lymph nodes induced tumor growth upon implantation. Two weeks after implantation, the weights of parathymic tumors were 1.5 and 2.0 g, respectively. For comparison, the weight of the parathymic lymph nodes in control rats was a significantly lower value (23.5±2.5 mg).

The ultimate proof of metastasis formation was provided in such a way that after tumor cell implantation, not the parathymic lymph nodes, but the left kidney was removed. As in previous experiments, it was assumed that if the lymph nodes contained metastatic cells, then the implanted lymph nodes would induce tumor growth under the renal capsule. Results corresponded to these expectations and to the observations of previous experiments. Parathymic lymph nodes implanted under the renal capsule after 1 or 3 days of nephrectomy were absorbed. To the contrary, lymph nodes removed 8 days after nephrectomy contained a sufficient number of metastatic cells to induce tumor growth after reimplantation (tumor weight: 6.5±0.9g). These experiments led to the conclusion that primary tumors containing 10^6 cells induced metastasis formation in about 3-6 days.

Mesenchymal mesoblastic nephroma (Ne/De)

Cell line establishment from mesoblastic nephroma and reimplantation measurements

The primary tumor pieces were digested with proteolytic enzymes, then the filtered and washed single cell suspension was placed into culture flasks at a cell density of 3 x 10^6. The doubling time of the adherent cells is 18-20 h. The absence of the contact inhibition and high grow rate are typical to these cells. The morphology and the ability of tumor and metastasis formation of the cells did not change over 100th passages. At the preconfluent stage of low cell density, the cells are randomly distributed (no islands), it was possible to distinguish among contracted bipolar and flattened polygonal cells. The cultured renal mesenchymal cells are small (7-10 µm), dense and granular when stained with Giemsa or hematoxylin and eosin. Seven days after the subcapsular implantation of Ne/De tumor cells (10^6), the left kidney was almost 50% larger, containing the distinguishable tumor, which infiltrated into the kidney causing its destruction. Often, a sharp demarcation line divided the healthy and neoplastic tissue.

In vitro uptake of radiotracer glucose analogue in Ne/De cells

Exponentially growing, daily subcultured cells were used for the *in vitro* uptake of glucose analogue. The incorporation of ^{18}FDG is approximately 3-times higher in mesenchymal renal tumor cells (Ne/De) than in control fibroblast cells. ^{18}FDG uptake indicates an aggressive growth rate and fast metabolism of Ne/De cell lines. In the presence of 5 µM Cytochalasin B, the ^{18}FDG incorporation was 7-times less in these cells.

Detection of GLUT transporters in Ne/De cell line by immunocytochemistry and Western blot analysis

Nephroblastoma (Ne/De) cells contain more transporters than fibroblasts. The localization of GLUT-1 and GLUT-3 was similar to that observed in HeDe cells. GLUT-3 expression was somewhat higher in NeDe cells than GLUT-1 expression. All results obtained with GLUT transporters correlated with results obtained in the Western blot analysis. GLUT-1 and GLUT-3 expression was the lowest in the control fibroblast cells. Tumor cell lines expressed higher relative levels of the GLUT transporters than control cells, as expected.

Detection of GLUT transporters in Ne/De tumor by immunohistochemistry and Western blot analysis

GLUT-1 level was equally low in the Ne/De cell line and in the mesenchymal kidney tumor. The expression of GLUT-3 was significantly higher in the Ne/De cell line and very high in the mesenchymal renal tumor. All results obtained with GLUT transporters correlated with results of the Western blot analysis, where we have found that the GLUT-3 and TGF-β1 levels were high with moderate expression of GLUT-1 transporter in the mesenchymal kidney tumor.

Metastatic potential of Ne/De cells followed by whole body autoradiography and phosphor image analysis and tissue distribution of ^{18}FDG

The metastatic tumor spread was supported by experiments related to tissue metabolism carried out in F344 rats. After 14 days of subcapsular transplantation of Ne/De cells, animals were given intravenously 15.0 MBq ^{18}FDG in saline. After 1 h animals were euthanized and – for the tissue distribution of ^{18}FDG measurements - blood samples were taken from the aorta. The tumor, liver, kidney, thymus and the abdominal rectal muscle and the parathymic lymph nodes were removed. Three tissue samples were taken from each organ and from parathymic glands and their activities were measured with a gamma counter. The DAR value of Ne/De tumor was 11-fold and the parathymic lymph nodes 9-fold higher than that of the muscle, supporting the notion that parathymic glands are involved in metastatic tumor growth. The DAR values of blood and other tissues of tumor-bearing animals did not differ significantly from those of control samples taken from healthy rats.

Autoradiographic experiments revealed that after 14 days, the majority of the radioactivity was accumulated in the renal pelvis and urinary bladder, where the radiotracer is excreted and accumulated before being removed by the urine. The radioactivity accumulated in the tumor, in the mesenteric lymph nodes and in the parathymic lymph nodes surpassed that of other organs. Even higher differences were obtained by phosphor image analysis. By taking the pixel intensity of resting striated muscle as one unit, the relative pixel densities were, in decreasing order: 20.78 in Ne/De tumor, 13.61 in parathymic lymph nodes, 6.29 in thymus, 5.15 in kidney, 3.06 in liver and 2.79 in blood. From these autoradiographic and phosphor image experiments, we concluded that Ne/De tumors grown under the capsule of kidney represent a significant metastatic burden manifested primarily in parathymic lymph nodes.

Appearance of metastases

Similaly to He/De cell implantation in the case of Ne/De, we also found that early implantation of lymph nodes of tumor-bearing animals (after one or three days) did not lead to tumor formation as the tumor spread takes more time and the implanted lymph nodes did not contain tumor cells, yet. Six days after Ne/De cell implantation, the isolated parathymic lymph nodes induced tumor growth upon implantation. The weights of the tumors 14 days after the implantation of six-day-old parathymic lymph nodes were 0.5 and 0.9 g. We concluded from these results that 10^6 cells containing primary tumors initiate metastasis formation between day 3 and 6 after implantation. It appeared that the slower growing He/De has a higher metastatic potential than the otherwise faster growing Ne/De tumor.

The ultimate series of experiments were modified in such a way that after tumor cell implantation, not the parathymic lymph nodes, but the left kidney was removed. Parathymic lymph nodes implanted under the renal capsule after 1 or 3 days of nephrectomy were absorbed. Lymph nodes removed 8 days after nephrectomy contained a sufficient number of metastatic cells to induce tumor growth after reimplantation (tumor weight: 3.0±0.9g).. Moreover, a significant difference was observed between the metastatic potential of He/De and Ne/De cells. These experiments led to the conclusion that primary tumors containing 10^6 cells induced metastasis formation in about 3-6 days. Although, we observed earlier that the primary tumor growth of He/De was always slower, its metastatic character turned out to be stronger than that of the faster growing Ne/De tumor.

Myelomonocytic leukemia (My1/De)

Cell line establishment from mesoblastic nephroma and reimplantation measurements

The single cell suspension from bone marrow of leukemic Long-Evans rats (80% myelomonocytic blast cells in bone marrow) was placed into culture flasks at a density of 3 x 10^6/ml. The doubling time of the suspension cells (100% blast cells) is 20 h. A high grow rate is characteristic to these blast cells, mitosis was frequently observed. This My1/De culture could be maintained for 2-2.5 months, as the blast cells differentiated into macrophages.

Two weeks after the transplantation of 10^6 My1/De blast cells under the renal capsule, the animals' liver (weighing often 10–15% of the body weight) and spleen became enlarged, the white blood cell number increased from the normal 6000/µl to 60000-80000/µl (40-80% blast cells). Histologycal examination revealed leukemic infiltration in parathymic lymph nodes (70% blast cells), in bone marrow (hypercellularity with 40-60% blast cells) and also in the liver and spleen. After 14 days of tumor cell implantation the tumor infiltrated into the kidney causing its destruction.

In vitro uptake of radiotracer glucose analogue in My1/De cells

Exponentially growing, daily subcultured myelomonocytic leukemia cells were used after the 20th passage for the *in vitro* uptake of glucose analogue. The ^{18}FDG uptake of these cells were compared with bone marrow suspensions, freshly isolated from control and My1/De sarcoma bearing Long-Evans rats. The incorporation of ^{18}FDG is approximately 3-times higher in blast cells (My1/De) than in control and leukemic bone marrow cells. ^{18}FDG uptake indicates an aggressive growth rate and fast metabolism of My1/De cells. The difference of ^{18}FDG uptake of bone marrow suspensions was not significant. In the presence of 5 µM Cytochalasin B, the ^{18}FDG incorporation was 9-times less in these cells.

Metastatic potential of My1/De cells followed by whole body autoradiography and phosphor image analysis and tissue distribution of ^{18}FDG

The *in vivo* distribution of ^{18}FDG was studied in tissues isolated from tumor-bearing Long-Evans rats. After the administration of 15.0 MBq ^{18}FDG the animals were euthanized, dissected and the differential absorption ratio (DAR) was determined in blood plasma, muscle, My1/De myelomoblastic sarcoma, liver, spleen, parathymic lymph node, thymus, bone marrow

and kidney. The DAR values were high – compared with the control - in the My1/De sarcoma, parathymic lymph node, liver and spleen. The DAR values of blood and other tissues of tumor-bearing animals did not differ significantly from those of control samples taken from healthy rats.

Rats were treated the same way as described above for the tissue distribution studies with the exception that tumor-bearing euthanized animals were not dissected. The radiotracer uptake was followed by whole body autoradiography. The rapid metabolic rates of leukemic (blast) cells are clearly visible in the primary tumor (myelomonocytic sarcoma) developed under the kidney, in the spleen and liver. As a unit of pixel density (radioactive counts/pixel) the resting muscle of the hind leg of the euthanized, tumor-bearing rat was chosen. By taking the relative pixel intensity of resting striated muscle as one unit, the relative pixel densities were the highest in the My1/De tumor, liver, spleen and in the parathymic lymph nodes.

DISCUSSION

We have established new cell lines from N-nitrosodimethylamine induced hepatocellular carcinoma (He/De) and mesoblastic nephroma (Ne/De). We have examined these cells *in vitro* and the reimplantation of the cells into experimental animals was successful. We obtained similar results with myelomonocytic leukemia cells, but these cells had a limited life span *in vitro*. By freezing and thawing these cells, their ability of proliferation, ability of tumor (primary and metastasis) formation did not change.

These cells were successfully implanted in experimental animals by a syngenic – heterotopic method. A new metastatic tumor model has been developed by inplanting tumor fragments or cells under the renal capsule. Heterotopic transplantation of cancer cells may take place by direct injection of tumor cells or surgical implantation (s.c.) of intact fragments of tumor. For many heterotopic models the use of surgical implantation of fragments improves the reproducibility and metastatic outcome of the tumor the model. Experimental metastasis may be caused by the injection of tumor cells directly to the systemic circulation. Depending on the site of injection and tropism of the tumor cell, distant metastases may (or may not) develop at a number of anatomic locations throughout the body within a short period. However, this approach does not allow the study of the early steps in the metastatic development and is a disadvantage of this model.

Our experiments have shown that chemically induced hepatocellular carcinoma (He/De), mesoblastic nephroma (Ne/De) and myelomonocytic leukemia (My1/De) tumor cells implanted under the capsule of the kidney generate metastases in the parathymic lymph nodes. This was confirmed by: 1) the implantation of ink particles under the capsule of the kidney which appeared within 24 h in the parathymic lymph nodes; 2) whole-body autoradiography and tissue distribution experiments of ^{18}FDG uptake, which showed ^{18}FDG accumulated in growing tumors and parathymic lymph nodes; 3) detection of overexpressed glucose transporters; 4) six days after tumor development (He/De, Ne/De), the removed and implanted parathymic lymph nodes behaved as solid tumors. These observations raise the question how the detached tumor cells get from the renal capsule to the parathymic lymph nodes. It is logical that the metastatic cells of tumors growing under the renal capsule enter first the lymphatic vessels of the diaphragm and then, primarily through the parasternal lymphatic vessels, reach the parathymic lymph nodes. We intended to develop a relatively isolated system to study the formation of metastases and to widen the range of available methods. One can of course argue that tumor growing under the capsule of the kidney may release metastatic cells to mesenteric lymph nodes as well, and detached tumor cells may reach the parathymic lymph nodes through the blood vessels, as refered to in the experiment of Blau. The anastomosis between the renal capsule and parenchymal lymph capillares is doubted, but has not been excluded, and this would change to some extent the localization of metastasis. Contrary to these objections, we regard the renal capsule-parathymic lymph node complex as an isolated system which provides an experimental approach to study angiogenesis and the malignant transformation of parathymic lymph nodes. Finally, our method is suitable for the experimental demonstration of chemical prevention of metastates formation.

We have shown an increased TGF-β protein level in the chemically induced tumors and metastases. Multiple investigators have suggested that the principal immune-suppressive factor secreted by tumor cells is TGF-β. Indeed it has been reported that the overexpression of TGF-β in the chemically induced tumors led to tumor growth and escape from immune-surveillance.

We have proved that there are significant differences in the chromatin condensation of different liver cells. Basic differences of potential diagnostic importance among chromatin structures of resting (Go), regenerating hepatocytes and hepatocellular tumor cells: 1. Nuclei of resting cells contain decondensed chromatin referred to as chromatin veil. Most of the open nuclei maintained their round shape or were only slightly elongated. 2. Nuclei of regenerating

cells opened up easily and showed a vide variety of chromatin structures, typical to logaritmically growing cells with most of the cells being in S phase. Most characteristic structures were chromatin ribbons. 3. Supercoiling was evident from the early stage of chromatin condensation referred to as veil-like chromatin in nuclei of hepatocarcinoma cells. The tendency of intensive supercoiling could be traced throughout the cell cycle. It was described by others that the length of the cell cycle in a fast-growing spontaneous mouse mammary tumor subline (11-12 h) was much shorter than that of the slow-growing spontaneous mouse mammary tumor subline (15-16 h). The difference in the growth rates for fast growing tumor cells versus slow growing or resting cells was attributable to the reduction of the length of the cell cycle in tumor cells. Failure of cell growth control is thought to play a major role in the development of tumors. We assume that in tumor cells the increased growth rate is directly related to the high activity of topoisomerases leading to the reduced length of cell cycle. These findings indicated that significant topological changes generated by elevated levels of topoisomerases may take place which were expected to have structural consequences at the chromatin level. The shortening of the cell cycle may cause mutations leading to cell death. Alternatively, the cause of apoptosis may be that rapidly growing tumors need to recruit more blood vessels to obtain nutritional requirements. Indeed, data indicate that growth-inhibition of tumor cells may induce apoptosis. It is logical to think that lagging angiogenesis, especially at the periphery of the growing tumor might cause lower O_2 levels (hypoxia) and drive external tumor cells into apoptosis.

SUMMARY

We have established new cell lines from N-nitrosodimethylamine induced hepatocellular carcinoma (He/De) and mesoblastic nephroma (Ne/De). The hepatocarcinoma (HeDe) and nephroblastoma (NeDe) cell lines and the tumors have been characterized with respect to their expression of GLUT-1 and GLUT-3 glucose transporters. We have examined the relationship between ^{18}FDG uptake and the expression of facilitative glucose transporters (GLUT-1 and GLUT-3). The higher ^{18}FDG uptake of tumor cells correlated with the GLUT-1 or GLUT-3 expression. Tumor cell lines expressed higher relative levels of GLUT transporters than the control cells. Significant differences were observed among the expressions of the tumors and the tumor cell lines.

Our experiments have shown that chemically induced hepatocellular carcinoma (He/De), mesoblastic nephroma (Ne/De) and myelomonocytic leukemia (My1/De) tumor cells implanted under the capsule of the kidney generate metastases in the parathymic lymph nodes. This was proved by the subcapsular implantation of clooidal ink particles, histopathology, immunohistchemistry, whole-body autoradiography and tissue distribution experiments of ^{18}FDG uptake. We regard the renal capsule–parathymic lymph node complex as an isolated system which provides an experimental approach to study angiogenesis and the potential role of parathymic lymph nodes in malignant transformation. Our system contributes to the understanding of: (a) the metastatic potential of rodent tumors; (b) the connection between the number of primary tumor cells and the temporal aspects of metastatic development. Finally our method is suitable for the experimental demonstration of chemical prevention of metastases formation.

We have found that the basic differences of potential diagnostic importance among chromatin structures of resting (Go), regenerating and hepatocellular tumor cells: 1. Nuclei of resting cells contain decondensed chromatin referred to as chromatin veil. Most of the open nuclei maintained their round shape or were only slightly elongated. 2. Nuclei of regenerating cells opened up easily and showed a vide variety of chromatin structures, typical to logaritmically growing cells with most of the cells being in S phase. 3. Supercoiling was evident from the early stage of chromatin condensation referred to as veil-like chromatin in nuclei of hepatoma cells. The tendency of intensive supercoiling in nuclei of tumor cells could be traced throughout the cell cycle.

NOVEL FINDINGS

1. The chemically induced hepatocellular carcinoma, mesoblastic nephroma and myelomocytic leukemia strains stored in liquid nitrogen are suitable for reimplantation under the renal capsule of inbred Fischer-344 and Long-Evans rats even after three years.

2. We have established new cell lines from hepatocellular carcinoma (He/De) and mesoblastic nephroma (Ne/De) tumors. We have examined these cells *in vitro* and the reimplantation of the cells into experimental animals was successful. We obtained similar results with myelomonocytic

leukemia, but these cells had a limited life span *in vitro*. By freezing and thawing these cells, their ability of proliferation, ability of tumor (primary and metastasis) formation did not change.

3. These results can be adapted in preclinical chemo-preventive examinations by using these rapidly growing tumor strains, which make the experiment time shorter. The use of exact cell number and the stability of the tumor strains or lines make experiments more reliable. The number of animal experiments can be drastically reduced.

4. We have proved that the ^{18}FDG, which is most commonly used in the medical imaging modality PET, is a useful tumor diagnostic tracer for chemically induced tumor bearing rats.

5. The chemically induced hepatocellular carcinoma (He/De), mesoblastic nephroma (Ne/De) and myelomonocytic leukemia (My1/De) tumor cells project metastases in the parathymic lymph nodes 6 days after their implantation under the capsule of the kidney. This method led to a reliable model for the observation of metastatic tumor formation and prevention.

6. We have proved that there are significant differences in the chromatin condensation of different liver cells. The nuclei of resting cells contain decondensed chromatin referred to as chromatin veil. The nuclei of regenerating cells opened up easily and showed a vide variety of chromatin structures. Supercoiling structure is tipical of the chromatin structure of hepatocarcinoma cells.

ANNEX

<u>This thesis built on the following publications:</u>

Trencsenyi G, Kertai P, Bako F, Hunyadi J, Marian T, Hargitai Z, Pocsi I, Muranyi E, Hornyak L, Banfalvi G. Renal capsule-parathymic lymph node complex: a new *in vivo* metastatic model in rats. Anticancer Res. 2009 Jun;29(6):2121-2126. **IF:** 1,390

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Banfalvi G, Klaisz M, Ujvarosi K, **Trencsenyi G**, Rozsa D, Nagy G. Gamma irradiation induced apoptotic changes in the chromatin structure of human erythroleukemia K562 cells. Apoptosis. 2007 Dec;12(12):2271-2283. **IF:** 3,043

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Gy. Trencsenyi, P. Kertai, F. Bako, J. Hunyadi, T. Marian, S. Kiss, G. Opposits, M. Emri, I. Pocsi and G. Banfalvi. Renal capsule parathymic lymph node complex: Imaging of a new i*n vivo* metastatic model in rats with ^{18}F-FDG. Nuclear Medicine Review, 2009, (12)1:39.

Gy. Trencsenyi, T. Juhasz, F. Bako, T. Marian, R. Salanki, P. Kertai, J. Hunyadi and G. Banfalvi. Comparison of tumorogenicity of liver and kidney tumors induced by N-nitrosodimethylamine by ^{18}FDG uptake and the expression of glucose transporters. Nuclear Medicine Review, 2009, (12)1:50.

T. Nagy, S. Kis, M. Emri, P. Mikecz, **Gy. Trencsenyi**, Z. Hendrik, G. Opposits, L. Trón, T. Marian. Investigation of the distribution of biologically active molecules in tissues of small animals using autoradiography and miniPET imaging. Nuclear Medicine Review, 2009, (12)1:51.

Trencsenyi Gy, Nagy T, Bako F, Hunyadi J, Emri M, Pocsi I, Banfalvi G, Marian T and Kertai P. A New *In Vivo* Metastatic Model In Rats: Renal Capsule - Parathymic Lymph Node Complex.
1st Central and Eastern Europian Laboratory Animal Conference (CEELA-2009), Budapest, 2009. (Poster)

Trencsényi Gy., Juhász T., Bakó F., Márián T., Salánki R., Kertai P., Hunyadi J., Bánfalvi G. N-nitrozodimetilamin indukálta máj és vese tumorok malignitásának in vitro vizsgálata ^{18}FDG felvétel és glükóz transzporterek expressziója alapján. 16th Congress of the Gyorgy Hevesy Society of Nuclear Medicine, Debrecen, 2009. (Poster)

Nagy T., Kis S.A., Emri M., Mikecz P., **Trencsényi Gy**., Hendrik Z., Opposits G., Trón L., Márián T. Pet radioligandok eloszlásának meghatározása laboratóriumi kisállatok szöveteiben autoradiográfiás és minipet módszerrel. 16th Congress of the Gyorgy Hevesy Society of Nuclear Medicine, Debrecen, 2009. (Poster)

Trencsényi Gy., Kertai P., Bakó F., Hunyadi J., Márián T., Kiss S., Opposits G., Emri M., Pócsi I. és Bánfalvi G.. A vesetok-parathymicalis nyirokcsomó komplexus – egy új *in vivo* metastasis modell vizsgálata ^{18}F-FDG radiopharmakonnal patkányokban. 16th Congress of the Gyorgy Hevesy Society of Nuclear Medicine, Debrecen, 2009. (Lecture)