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Current Status Review

Multicellular spheroids: a three-dimensional *in vitro* culture system to study tumour biology

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Summary. The growth of tumour cells as three-dimensional multicellular spheroids *in vitro* has led to important insights in tumour biology, since properties of the *in vivo*-tumour such as proliferation or nutrient gradients, can be studied under controlled conditions. While this review starts with an update of recent data on spheroid monocultures, especially concerning tumour microenvironment and therapeutic modalities, the main emphasis is put on the spectrum of heterologous cultures which have evolved in previous years. This type of culture includes tumour cell interaction with endothelial, fibroblast or immunocompetent cells. The relation of the spheroid culture model to other types of three-dimensional culture and our critical evaluation and presentation of the technical aspects of growing and analysing spheroids are included in the text. These topics are chosed to help the experimental pathologist design experiments with tumour spheroids and to stimulate discussion.

Keywords: Multicellular spheroids, *in vitro*, three-dimensional culture system, tumour

Introduction

Over the past three decades three-dimensional (3-D) cell cultures of both normal and malignant origin have been progressively used as *in vitro* systems in organogenesis and biomedical research. In contrast to conventional, homogeneous monolayer or suspension cultures, three-dimensional cultures may preserve specific biochemical and morphological features similar to the corresponding tissues *in vivo* and can stay in a differentiated, active functional state for many weeks. A major advantage of establishing three-dimensional, spherical aggregates from permanent cell lines is that basic mechanisms of cell growth, proliferation, and differentiation can be studied in a reproducible format with an internal environment dictated by the metabolism and adaptive responses of the cells. Thus, aggregates possess a three-dimensionally organized complex network displaying cell-to-cell and cell-to-matrix interactions in conjunction with a well-defined morphological and physiological geometry affecting internal criteria such as cell shape, size, distribution of organelles, or enzymatic activity. This 'secular heterogeneity' (Heppner & Miller 1989) should be distinguished from clonal cell heterogeneity and is reflected in 3-D rather than 2-D culture systems.

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Since its first introduction in the early seventies by Sutherland and coworkers (Sutherland *et al.* 1970a,b; Inch *et al.* 1970), multicellular tumour spheroids (MCTS) have gained fundamental importance in experimental cancer research with the vast majority of previous studies focusing on tumour cell response to various treatment modalities (for reviews see: Mueller-Klieser 1987; Sutherland 1988; Knuechel & Sutherland 1990; Carlsson 1992; Carlsson & Nederman 1992; Freyer 1992). In addition to therapeutically orientated investigations, the use of MCTS has expanded to other areas of basic cell biology and pathophysiology such as intercellular communication, cell invasion, angiogenesis, and neovascularization (reviewed by: Bracke *et al.* 1992; Engebraaten 1992; Neeman *et al.* 1997). Similarly, investigations on microenvironmental and epigenetic mechanisms involved in the regulation of cell proliferation, differentiation, cell death, and gene expression have been intensified within the last 10 years (Acker & Carlsson 1992; Knuechel & Sutherland 1992; Mueller-Klieser 1997).

Although 3-D culture systems such as multicellular tumour spheroid monocultures are considered to mimic the *in vivo* micromilieu of small avascular tumours, much of the biological complexity of the *in vivo* situation is lost. In organogenesis for example, numerous reports have been published dealing with the role of mesenchyme and its extracellular matrix. It is well known that fibroblastic mesenchyme is not only critical to the development of the integumental, urinary, gastrointestinal, skeletal and urogenital systems pre- and postnatally but also induces specific patterns of epithelial morphogenesis and cytodifferentiation such as the expression of tissue specific secretory proteins (for review: Cunha *et al.* 1991). In addition, the intricate macromolecular network of the ECM which is predominantly produced and secreted by stromal cells has been shown to play a crucial role in cell/epithelial differentiation and tissue-specific gene expression acting as positive or negative regulator. For parenchymal organs the microenvironment can thus be defined as a unit of function consisting of the epithelium, its underlying basement membrane, and the subjacent stroma. Nevertheless, fibroblasts have been investigated as targets of tumour cell invasion rather than in tumour cell differentiation. The possibility that emerging or established carcinomas may be regulated by their connective tissue environment has come into focus only recently.

In addition to endothelial cells which are mainly involved in tumour angiogenesis and neovascularization (for review: Folkman 1990; Mareel *et al.* 1990; Bracke *et al.* 1992), tumour-associated immunocompetent cells represent an important cell population to be considered as negative or positive affectors of tumour cell growth and propagation (Mantovani 1992).

Besides recent data on classical topics of research on MCTS monocultures, this review will highlight work with heterologous cocultures. An overview on the technical aspects of working with homologous and heterologous cultures is presented to provide insight into the practicality of the tissue culture method. We intend to show that a three-dimensional controlled model system, including both normal and tumour cells and their products will offer understanding of tumour micromilieu appropriate to test new anticancer strategies *in vitro*, allowing for two therapeutic targets, the tumour cell and its environment.

Spheroid monocultures

Technical aspects I

Diverse techniques for cultivation of tumour spheroids have been described in the literature (for review: Mueller-Klieser 1987; Lund-Johansen *et al.* 1992). Spinner flask cultures are most widely used since large numbers of spheroids may be generated at the same time reaching diameters up to 1–2 mm. Other approaches to avoid effects caused by stagnant medium are roller bottles or roller tubes. With all methods spherical aggregates of tumour cells form spontaneously in the absence of attachment to another substrate either directly in the flasks or during an initiation interval in agar-coated or microbacteriological culture dishes. For rapid harvesting of spheroids of approximately the same size, cultures may be pipetted through a sieve of nylon meshes prior to the transfer into spinner flasks or prior to the final experiment. Thus, one can easily culture large numbers of spheroids to obtain precise growth data while each individual spheroid is essentially identical to others in the complex, yet ordered, internal physiology and morphology. However, for monitoring and manipulating single spheroids at particular growth stages, cultivation of aggregates on a stationary, nonadherent surface such as agar or agarose utilizing 96-well plates is recommended. These so-called agar or liquid overlay cultures may be kept on a gyratory shaker following a defined initiation time in order to guarantee optimum nutrient supply. In 1990, Koide *et al.* (1990) described a novel, semiadhesive substratum method for spheroid cultivation taking advantage of the spontaneous detachment of small aggregates formed on semiadhesive substrates such as proteoglycans or positively charged polystyrene.

Spheroid diameters to estimate spheroid volume

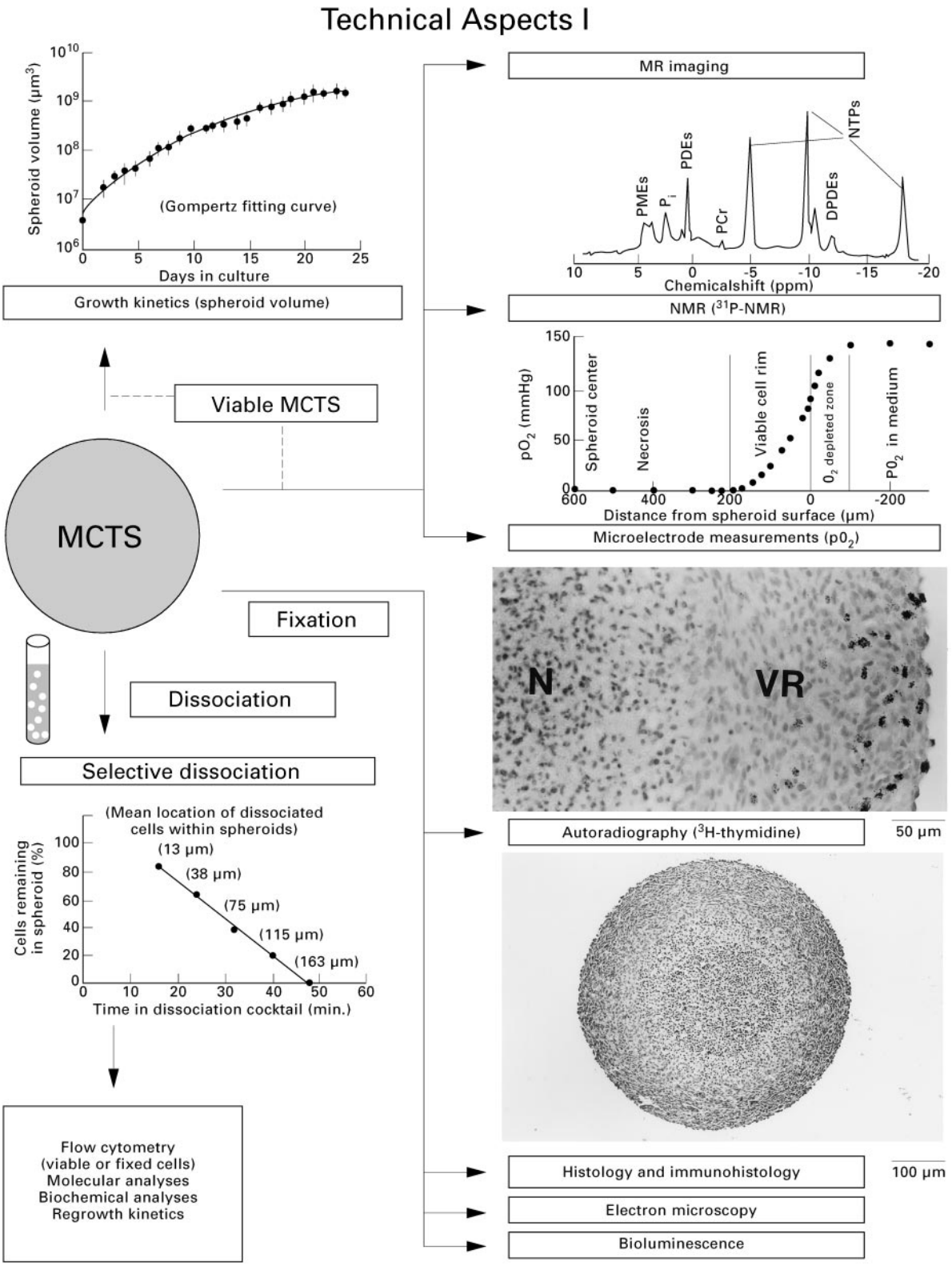
growth curves are conventionally measured with a reticule manipulated by a micrometer screw in the eyepiece of an inverted microscope. More automatic methods for measurements of growth curves or for size-dependent sorting of spheroids are based on microscope-image-analyser, flow cytometry or laser-diffraction particle sizer. The Gompertz equation first introduced by Winsor (1932) has been shown to mathematically describe not only early tumour growth as summarized in the early 60s by Laird (1964, 1965) but has also been applied to a variety of spheroid types in the literature (e.g. Marusic *et al.* 1994; Kunz-Schughart *et al.* 1996). In previous studies, influences of growth-promoting or growth-suppressing agents have been mainly described by disturbances in growth curves quantified as growth delay. However, promising attempts have also been made to connect data from growth curves with data from single-cell survival experiments by different calculation methods raising some hope for the future work with growth curves as discussed to some detail by Carlsson & Nederman (1992). In addition to volume growth kinetics, analysis of the proliferative activity has become an important tool in spheroid research. The proliferative status can be determined via autoradiography using ^{3}H-thymidine (TLI, thymidine labelling index), immunohistological staining utilizing the BrdUrd antibody technique or antibodies detecting proliferation-associated antigens such as Ki-67 or PCNA, or via flow cytometry following staining of dissociated, fixed or unfixed spheroid cells with DNA-specific fluorochromes such as Hoechst 33342, propidiumiodide, or mithramycin.

Although the three-dimensional structure of spheroids limits the study of single cells *in situ*, several technical approaches enable a morphological and physiological as well as biological and molecular characterization of spheroids (i.e. Figure 1). Frozen or chemically fixed spheroid sections can be studied for antigenic differences via selective immunological labelling visualized by fluorescent agents or enzymatic techniques such as peroxidase or alkali phosphatase using the light microscope. Transmission electron microscopy on ultrathin spheroid sections allows for the investigation of the ultrastructure of spheroids and scanning electron microscopy has been used to investigate cell and tissue surfaces. Spheroid physiology can be determined *in situ* not only by microelectrode techniques such as for pO_2 or pH measurements (e.g. Mueller-Klieser 1984, 1987; Acker *et al.* 1987ab; Acker & Carlsson 1992; Kunz-Schughart *et al.* 1996) but can also be monitored using nuclear magnetic resonance spectroscopy, e.g. to study high energy phosphates or lipid metabolism (Freyer *et al.* 1990, 1991; Ronen *et al.* 1992), or magnetic resonance microimaging, i.e. to provide information on intracellular water-diffusion coefficients in individual MCTS (Sillerud *et al.* 1990; Neeman *et al.* 1991). Other methods such as the Lowry technique or bioluminescence used to investigate steady state levels of ATP, lactate, and glucose have to be carried out on shock-frozen/fixed spheroid sections (Walenta *et al.* 1990; Teutsch *et al.* 1995). Since the sensitivity of the latter methods is clearly beyond cellular level, selective spheroid dissociation prior to single cell analysis by flow cytometry, high-performance liquid chromatography or molecular techniques has become an important prerequisite for the correlation of an isolated cell population with its initial location in the aggregate. Diverse techniques for cell isolation from spheroids have been reviewed by Freyer & Schor (1989) who established an improved, automated dissociation procedure based on the sequential exposure of spheroids to a trypsin solution which can be modified for a variety of spheroid types (e.g. Kunz-Schughart & Freyer 1997). Figure 1 summarizes the most convenient techniques that have been applied to characterize the (patho-)physiological behaviour of cells in spheroid culture showing some representative data determined within large ras-transformed rat embryo fibroblast spheroids (for more detail: Kunz-Schughart *et al.* 1996, 1997).

Microenvironment

Beyond a critical size ($\geq 500\,\mu$m), most spheroids from permanent cell lines develop a necrotic core surrounded by a viable rim of cells (100–300 μm) consisting of proliferating cells in the spheroid periphery and quiescent, yet intact and viable cells close to the necrotic center. Cell organization and thickness of the viable cell layers significantly differ for different cell types and also depend upon the spheroid cultivation method. However, the concentric arrangement of heterogeneous cell populations in spheroids as well as their growth pattern clearly mimic initial, avascular stages of solid tumours *in vivo*, not-yet-vascularized micrometastatic foci, or intercapillary tumour microregions with high proliferative activity close to the capillaries, quiescent cells as intermediates, and necrotic areas at larger distances (for review: Mueller-Klieser 1987; Sutherland 1988).

Several investigators have tried to link development of necrosis and growth arrest in the spheroid system to deficiencies in energy-related metabolites. For some spheroid types such as WiDr human colon adenocarcinoma and tumourigenic Rat1-T1 rat embryo fibroblasts a coincidence of emergence of necrosis and hypoxia during growth has been documented with the thickness


Technical Aspects I
Spheroid volume (µm³)
(Gompertz fitting curve)
Days in culture
Growth kinetics (spheroid volume)
MR imaging
PMEs
Pi
PDEs
PCr
DPDEs
NTPs
Chemicalshift (ppm)
NMR (31P-NMR)
pO2 (mmHg)
Spheroid center
Necrosis
Viable cell rim
O2 depleted zone
PO2 in medium
Distance from spheroid surface (µm)
Microelectrode measurements (pO2)
Viable MCTS
MCTS
Fixation
Dissociation
Selective dissociation
(Mean location of dissociated cells within spheroids)
(13 µm)
(38 µm)
(75 µm)
(115 µm)
(163 µm)
Cells remaining in spheroid (%)
Time in dissociation cocktail (min.)
N
VR
Autoradiography (3H-thymidine)
50 µm
Flow cytometry
(viable or fixed cells)
Molecular analyses
Biochemical analyses
Regrowth kinetics
Histology and immunohistology
100 µm
Electron microscopy
Bioluminescence

of viable cell rim reflecting oxygen availability (Kunz-Schughart *et al.* 1996; Monz *et al.* 1996). Theoretical considerations by Groebe & Mueller-Klieser 1996) suggest that a single limiting factor such as oxygen deficiency might explain the development of necrosis. However, there is some evidence that necrosis in MCTS is a multifactorial event affected by lack of oxygen and/or nutrients, accumulation of waste products, and low pH as discussed by Acker and colleagues (Acker *et al.* 1987ab; Carlsson & Acker 1988). Independent studies by Mueller-Klieser and associates (Bredel-Geissler *et al.* 1992; Teutsch *et al.* 1995; Walenta *et al.* 1990) and Freyer *et al.* (1990, 1991) on steady-state levels of glucose, lactate, and energy-rich phosphates in EMT-6 mouse mammary carcinoma spheroids indicate that cells in the inner spheroid regions may adapt their metabolism to environmental stress maintaining intracellular homeostasis until shortly before cell death; thus, critical reduction in ATP and intracellular pH can be excluded as a mechanism for cell quiescence and necrotic cell destruction.

Estimations of oxygen consumption rates from oxygen partial pressure gradients within spheroids (i.e. Figure 1) imply a uniformly distributed cellular respiratory activity (Bourrat-Floeck *et al.* 1991; Groebe & Mueller-Klieser 1991; Mueller-Klieser 1984; Mueller-Klieser *et al.* 1985), while selective dissociation of the same spheroid types followed by *in situ* oxygen consumption measurement showed a reduction in oxygen uptake per cell as a function of depth within the spheroid (Freyer 1994). Morphometric studies by Bredel-Geissler *et al.* (1992) document a relatively constant number of mitochondria across the viable cell rim of spheroids, yet, without taking into account their functional status. Recent flow cytometric analyses of mitochondrial mass and function confirmed this observation and clarified that mitochondrial activity which may influence oxygen consumption seems to decrease as a function of the cell cycle distribution in spheroids being highest in the spheroid periphery (Kunz-Schughart *et al.* 1997). However, it remains speculation if cell quiescence and accumulation of G_0-phase cells in spheroids are accompanied by or result from reduction in mitochondrial function and oxygen uptake.

Rak *et al.* (1995) have demonstrated that 3-D growth conditions not only result in central necrosis and cell cycle arrest but may induce massive programmed cell death in spheroids of intestinal epithelial cells. Growth inhibition via stimulation of apoptosis has also been shown by Fujiwara *et al.* (1993, 1994) for p53-defective human lung cancer spheroids transfected *in situ* with a viral wild-type p53 vector resulting in the level of subsequent gene expression being proportional to induction of apoptosis. Mueller-Klieser (1997) recently reported the development of apoptosis of multinucleated giant cells in a highly differentiated rhabdomyosarcoma spheroid type and in undifferentiated V79 hamster lung aggregates as visualized by immunostaining of DNA fragments using the TUNEL assay. There is some evidence that microenvironmental stress such as hypoxia reflected by a transient increase in glutathione may play a crucial role in the accumulation of apoptotic cells in V79 spheroids (Romero *et al.* 1997).

Differentiation in spheroid culture includes not only conservation of morphogenic capacities and histiotypic reorganization but also maintenance of functional activities (for review: Knuechel & Sutherland 1992). Differentiation pattern such as neural rosette formation in teratocarcinoma spheroids (Kawata *et al.* 1991) or generation of myotube-like, multinucleated giant cells in the central regions of large, non-necrotic spheroids from clonal human rhabdomyosarcoma cells have been described previously (Karbach *et al.* 1992). Also, epithelial organization within different squamous carcinoma spheroid types associated with modified deposition and/or distribution of biomatrix such as extracellular matrix molecules fibronectin, collagens I, III and IV or laminin have been documented (Knuechel *et al.* 1990). Waleh *et al.* (1994) examined the function of ECM receptors in large, EGF-dependent transformed A431 spheroids. Integrin subunits α6, β1, and β4 were reduced in spheroids as opposed to monolayers shown on the protein as well as on the mRNA level. These results together with the studies by Paulus *et al.* (1994) and

Figure 1. Technical Aspects I: Spectrum of analytical technology to characterize MCTS monocultures. In addition to spheroid volume growth, a selection of representative measurements carried out in individual spheroids or spheroid populations of oncogene-transfected rat embryo fibroblast spheroids (Rat1-T1) with a diameter of 1200-1500 μm is shown. From above: ^{31}P-NMR (nuclear magnetic resonance) spectrum showing PMEs (phosphomonoesters), P_i (inorganic phosphate), PDEs (phosphodiesters), PCr (phosphocreatine), NTPs (nucleosidtriphosphates), and DPDEs (diphosphodiesters). The idea of NMR measurements on MCTS is to noninvasively determine parameters influencing and/or reflecting steady-state cellular metabolism such as energy-rich phosphates, membrane phospholipid precursors and degradation products, etc. Further, a pO_2 gradient from the spheroid periphery towards the spheroid center using a Whalen-type oxygen-sensitive microelectrode, an autoradiograph (^{3}H-thymidine labelling), a conventional H & E-stained histological image (median section), and a selective dissociation experiment are shown. MR, magnetic resonance; pO_2, oxygen partial pressure; N, necrosis; VR, viable cell rim.

Hauptmann *et al.* (1995) reveal that spheroids mimic the pattern of integrin expression found *in vivo* indicating that cell-cell contact and microenvironment regulate expression and distribution of a subset of integrin molecules. In an intriguing quantitative study using breast and colon carcinoma spheroids, Byers *et al.* (1995) showed that intact E-cadherin expression and proper linkage of this transmembrane molecule to the cytoskeleton is an essential factor for a strong cell-cell adhesion in 3-D culture.

Maintenance of cell function in 3-D as opposed to 2-D cultures has also been studied by Huelser *et al.* demonstrating that gap-junctional communication in small aggregates is similar to monolayer cells but clearly down-regulated in larger spheroids (for review: Huelser 1992). Connexin expression and intercellular communication was investigated in human bladder carcinoma spheroids by Knuechel *et al.* (1996) demonstrating discrepancies between monolayer and spheroid cultures which may be due to the modifications in the differentiation pattern. A series of experiments from the laboratory of Sutherland and associates showed that human squamous carcinoma cells A431 that were more differentiated in spheroids than in monolayers were characterized by a three-fold higher transcription of TGF-α and a two-fold higher TGF-α protein expression as well as increased heme oxygenase expression while EGF-receptor at the cell surface was remarkably reduced (Knuechel *et al.* 1990; Laderoute *et al.* 1992; Mansbridge *et al.* 1992). In contrast, growth of A431 cells was stimulated by EGF (epidermal growth factor) in spheroids, but not in monolayers (Murphy *et al.* 1993). A particularly interesting study has been published by Theodorescu *et al.* (1993) who compared the time course of TGF-β1 transcription affected by exogenous, active TGF-β1 protein in MDA-MB-231 human breast carcinoma and A549 human lung carcinoma cells in monolayer and spheroid culture. In A549 spheroids, exogenous TGF-β1 did not alter the steady state level of TGF-β1 transcripts while it stimulated transcription in monolayers. Vice versa, TGF-β1 protein was ineffective in MDA-MB-231 monolayers but enhanced TGF-β1 transcription in spheroids. Kluender & Huelser (1993) demonstrated the effects of spheroid culturing on the regulation of a transfected gene. Essentially all monolayer cells transfected with a lacZ reporter gene under the control of actin promoter express β-galactosidase while most of the cells are negative when grown in spheroid culture for more than one week. This effect was reversible indicating that 3-D culturing profoundly affects regulation of gene expression. Similarly, it has been documented that expression of specific angiogenic factors such as VEGF may be induced by the three-dimensional milieu probably due to microenvironmental stress in human colon carcinoma and glioma spheroids (Acker *et al.* 1990; Shweiki *et al.* 1995; Waleh *et al.* 1995).

Therapeutic strategies

First attempts to use 3-D aggregates for experimental cancer therapy trace back to the early seventies when MCTS were applied in radiobiology research. Several detailed reviews summarize the basic insights into radiation response of tumour cells advanced with the spheroid model such as the cell-cell contact effect which might be correlated with the degree of electrical cell-to-cell coupling (Huelser 1992) and the role of microenvironmental factors in controlling radiation survival, e.g. correlation between quiescent cells, hypoxia, and radioresponsiveness (Mueller-Klieser 1987; Sutherland 1988; Knuechel & Sutherland 1990; Freyer 1992). As a result, only a few studies on conventional aspects of radiotherapy have been published within the last five years such as the work by Reddy *et al.* (1994) describing the absence of a radioprotective contact effect in spheroids of different chinese hamster sublines or the studies by Frank *et al.* (1993) and Fritz *et al.* (1996) who analysed survival, cell cycle effects, DNA lesions and repair in V79 monolayer and spheroid cells after irradiation at high- and low-dose rate or superfractionation regime. A relatively new series of investigations focusing on radiosensitivity, DNA repair capacity, and stem cell fractions of various human spheroid types after fractionated irradiation has been published by Stuschke *et al.* (1992a,b, 1993a,b, 1995) discussing the degree of cell differentiation in spheroids as relevant parameter of radioresponsiveness.

In addition to experimental radiotherapy, spheroids have been increasingly applied in anticancer drug testing. As for radioresponsiveness, drug effect was mainly quantified by analysis of spheroid volume growth delay, clonogenic survival capacity and/or outgrowth tests. The latter represents an alternative, where cells are allowed to migrate out of the spheroid and form a growing monolayer (for review: Mueller-Klieser 1987; Sutherland 1988; Carlsson & Nederman 1989). It is of specific interest that multicellular-mediated resistance similar to the contact effect in radiotherapy has been reported for some anticancer agents (most recently: Kobayashi *et al.* 1993; Graham *et al.* 1994) indicating a basic molecular mechanism as discussed by Olive & Durand (1994). Drugs tested so far include alkylating agents such as nitrosurea substances, chlorambucil or melphalan, antibiotics, e.g. adriamycin, daunomycin or bleomycin, as well as plant alkaloids namely vincristine,

vinblastine or etoposide. Several other previous studies deal with the action of antimetabolites such as 5-fluorouracil or methotrexate, cisplatin, flavone acetic acid and/or retinoic acid. In addition to novel chemotherapeutic agents like synthetic thymidylate synthase inhibitors (Banks *et al.* 1994) or the active vitamin-D metabolite 1,25-dihydroxyvitamin D_3 (Brackman 1995), combination therapy should be further exploited by using multicellular spheroids. For example, Sacks *et al.* (1995) have shown in squamous carcinoma spheroids, that retinoic acid-growth inhibited cells characterized by continuing DNA synthesis exhibit increased sensitivity to cisplatin and 5-fluorouracil.

Establishment of new chemotherapeutic attempts, in particular the coadministration of different drugs and/or antibody-carrying toxic radionuclides requires better understanding of how metabolic gradients and heterogeneity influence therapeutical outcome. Differences in toxic action that are obtained at different spheroid locations may be due to modifications in the micromilieu of the cells, e.g. in the central hypoxic, nutrient starvation zone, but may also result from penetration problems (Knuechel *et al.* 1989). Techniques used to investigate substance penetration are conventional histology/autoradiography for drugs that are bound to cellular or extracellular structures, oil centrifugation, liquid scintillation counting, and a freeze-drying method to preserve distribution of nonbound drugs in spheroids as reviewed by Carlsson & Nederman (1992). While drugs like 5-fluorouracil, cisplatin, or chlorambucil can easily penetrate into spheroids, the poor effect of vinblastine could, to a large extent, be explained by very slow penetration.

In addition to conventional therapeutic strategies including hyperthermia (for review: Schem & Dahl 1992), spheroids have been involved in studies on the mechanism of photodynamic treatment and diagnosis utilizing photocytotoxic agents such as aluminium phthalocyanine species, photofrin or more recently the hemeprecurosr 5-aminolevulinic acid [ALA] (e.g. Chan *et al.* 1991; Foster *et al.* 1993; West & Moore 1992; Steinbach *et al.* 1994). The photosensitizing substance protoporphyrin IX, which accumulates intracellulary after exposure to ALA, is found in epithelial cells, however, hardly in stromal cells of patient samples. This finding can be mimicked with heterologous spheroids of fibroblasts and tumour cells, however, is different in monolayers of the same cells (Steinbach *et al.* 1995).

An additional series of investigations concentrates on the subject of radio-immunotherapy. Penetration, distribution in a heterogeneous micromilieu, and efficacy of diverse radionuclides used to label new types of radiopharmaceuticals such as ^{131}I-meta-iodobenzylguanidine (Gaze *et al.* 1992; Mairs *et al.* 1995) or ^{125}I- or ^{131}I-labelled antibodies (Bardiès *et al.* 1992; Essand *et al.* 1995) have been measured. The authors are convinced that estimations of radionuclide uptake and retention kinetics as well as antibody/antibody-fragment binding and penetration in spheroids *in vitro* provide important and prospective information on suitability and practicability of radionuclide treatment and antibody-mediated targeting in cancer therapy *in vivo* (for review: Carlsson 1992; Walker *et al.* 1988; Wheldon 1994).

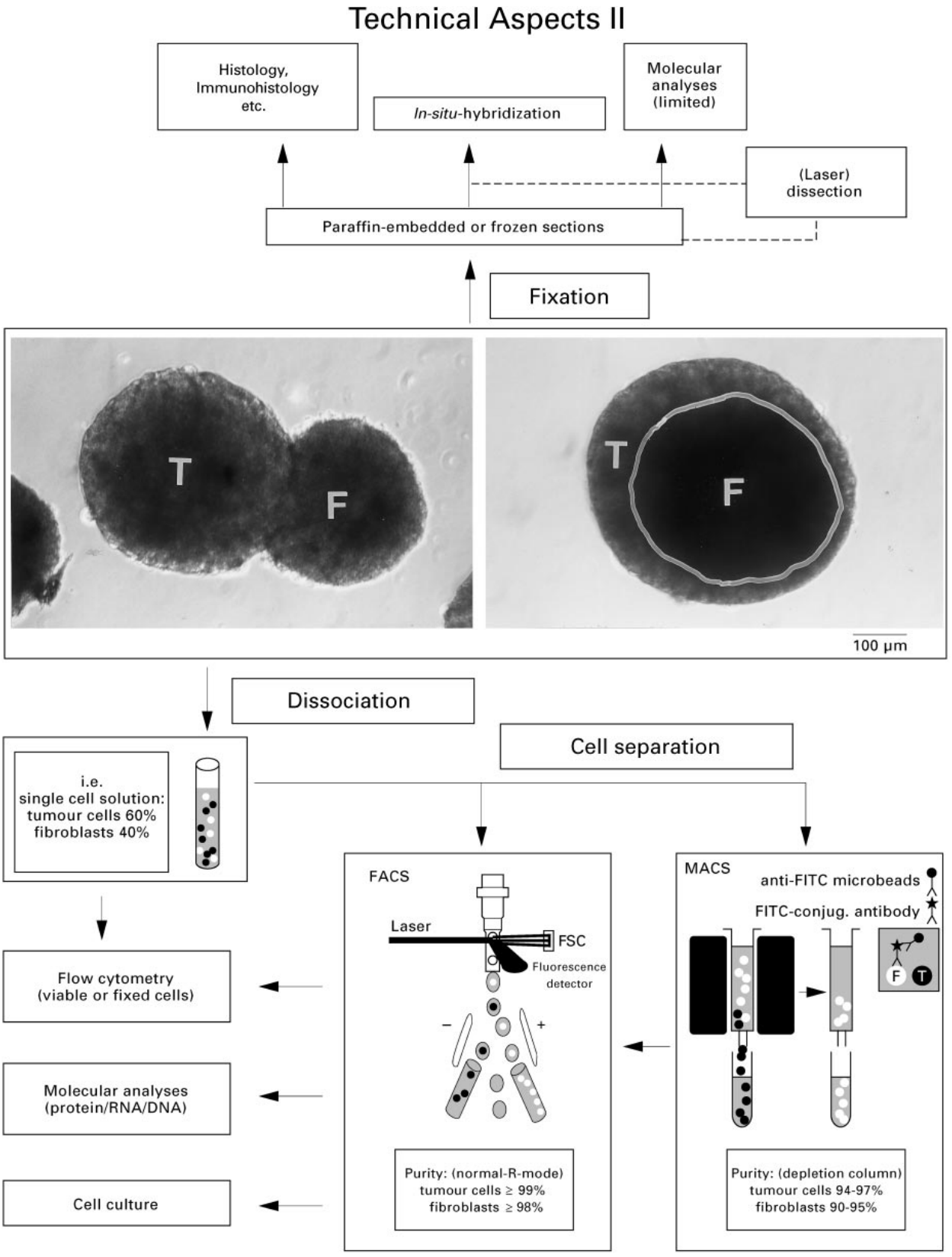
One major reason for the development of spatial distributions of chemotherapeutic drugs and heterogeneous therapeutical effects of diverse anticancer treatments is the establishment of starvation and hypoxic zones in tumours *in vivo* which is reflected in MCTS (see Microenvironment chapter). Recent progress to quantify the proportion of hypoxia in tissue has been made by the development of novel hypoxia markers and by the establishment of the 'comet assay'. In addition to a detailed discussion on hypoxia and its induction by chemical agents in spheroids by Freyer (1992), new promising studies on this subject utilizing spheroids have been highlighted by Mueller-Klieser (1997).

Spheroid cocultures

Technical aspects II

With the coculture of more than one cell type in spheroids important aspects of tumour cell interactions can be modelled under standardized conditions. The higher level of complexity involves new challenges for analytical techniques, which are similar to those of primary tumour material. Mostly, fixation of cocultures followed by immunohistochemical staining of sections is applied as routine method. While the detection of antigen presence and distributions in cocultures is possible, sensitivity of immunohistology is rather low as opposed to flow cytometric or molecular analyses. Frozen or chemically fixed and paraffin-embedded sections of cocultures may also be investigated using *in situ* hybridization or purification of nucleic acids for further limited molecular analysis. Separation of fixed cells from defined locations within these sections can be obtained by using microdissection techniques, e.g. via laser microbeam, probably combined with an optical tweezer as already applied for tissue (e.g. Becker *et al.* 1996; Schuetze & Clement-Sengewald 1994).

In order to investigate molecules involved in the complex interplay of different cell types, routine molecular techniques such as diverse blotting procedures may be useful. Low amount of material (poor cell count or DNA/


Technical Aspects II
Histology, Immunohistology etc.
In-situ-hybridization
Molecular analyses (limited)
(Laser) dissection
Paraffin-embedded or frozen sections
Fixation
T
F
T
F
100 µm
Dissociation
Cell separation
i.e. single cell solution: tumour cells 60% fibroblasts 40%
Flow cytometry (viable or fixed cells)
Molecular analyses (protein/RNA/DNA)
Cell culture
FACS
Laser
FSC
Fluorescence detector
Purity: (normal-R-mode) tumour cells ≥ 99% fibroblasts ≥ 98%
MACS
anti-FITC microbeads
FITC-conjug. antibody
F
T
Purity: (depletion column) tumour cells 94-97% fibroblasts 90-95%

RNA abundance) often requires more sensitive methods, e.g. enhancement via PCR (polymerase chain reaction). Pure populations of viable cells have to be used, in particular to screen cocultures for differential gene expression using highly sensitive techniques such as DD-PCR (differential display PCR), AP-PCR (arbitrarily primed PCR), or subtractive and competitive hybridization. Following dissociation of cocultures by enzymatic and/or mechanic means, fluorescence-activated (FACS) and/or magnetic cell separation (MACS) to isolate different cell populations for further molecular analysis is recommended (Figure 2). To briefly summarize the authors experience in cell sorting of tumour cells and monocytes/macrophages or fibroblasts, it is emphasized that MACS (Miltenyi/Biotec, Bergisch Gladbach, Germany) using antibodies conjugated with super-paramagnetic microbeads is an easy-to handle, rapid, and 'gentle' cell separation technique. However, due to a relatively low purity of the sorted cell fractions (< 98%) which highly depends upon the ratio of the different cell types in coculture, it should be used primarily for cell enrichment. In contrast, cell viability is rather influenced by FACS than MACS, and sterile sorting of high cell numbers is more time-consuming. The sort quality and purity of cell fractions separated by FACS is significantly higher allowing for some molecular analyses.

As a prerequisite for detection and separation of different cell types in 3-D culture, cell subpopulations may permanently be labelled by introduction of specific marker genes (genetic marking) such as the β-galactosidase or other bacterial genes (e.g. Bradley & Pitts 1994; Frenzel *et al.* 1995). A unique method for the fluorescent labelling of viable mammalian cells is the transient (or stable) transfection of one cell type with the Aequorea victoria gfp DNA leading to the expression of GFP (green fluorescence protein) which can easily be detected via flow cytometry using 488 nm excitation as well as in a fluorescence microscope (e.g. Chalfie 1995; Cubitt *et al.* 1995; Lybarger *et al.* 1996). Recent, intensive research in GFP engineering clearly improves its feasibility (e.g. Heim & Tsien 1996) and GFP transfection should be considered for labelling of cell subpopulations in coculture systems. Another direct method for the detection of specific cell subpopulations that do not clearly differ in cell surface antigen presentation is the long-term tracking of cell membranes using PKH (P.K. Horan) markers (Teare *et al.* 1991) Promising methodological research efforts in cell marking and separation followed by molecular analyses will in general extent the availability of heterologous *in vitro* coculture systems in the near future.

Figure 2. Technical Aspects II: Spectrum of analytical technology to characterize spheroid cocultures. Photographs show cocultures of BT474 breast carcinoma spheroids and N1 fibroblast aggregates after 2 (left) and 8 (right) days in coculture. The initial size of spheroids prior to cocultivation was 300-400 μm for BT474 MCTS and 250-350 μm for N1 aggregates. White line indicates contact zone of fibroblast aggregates and tumour cells. Analysis of single cells or cell suspensions by modern molecular/biochemical techniques requires dissociation of cocultures followed by separation of different cell subpopulations., i.e. by MACS and/or FACS. F, fibroblasts; T, tumour cells; MACS, magnetic cell separation; FACS, fluorescence activated cell separation; FSC, forward light scatter; FITC, fluorescein isothiocyanate.

Tumour modelling

In order to understand the role heterologous spheroids have as a model system in tumour biology, an update on related alternative models is given in this chapter. Tumour organ cultures, as well as reaggregation cultures of normal or tumour cells will be discussed. Also the tendency to use culture systems with defined artificial matrices, mostly used for normal cells, seemed worth reviewing to some extent, since data on the degree of cellular differentiation in these three-dimensional systems are provided.

A variety of primary tissues can be maintained in organ culture with histiotypic organization for a limited period of time. Based on this observation, an elegant method for long-term cultivation *in vitro* of tumour biopsy specimens with tumour-specific cellular complexity and differentiation pattern was established (for review: Bjerkvig *et al.* 1992; Fjellbirkeland *et al.* 1995). One advantage of these so-called 'tumour fragment spheroids' over conventional spheroid cultivation techniques is a remarkable stability in tumour cell ploidy while clonal evolution associated with genetic alterations has been shown for the same cells kept in monolayer culture. Paulus *et al.* (1994) described a distinct expression pattern for ECM proteins and their integrin receptors in fragment cultures as opposed to reaggregation spheroids using immunohistochemical staining and RT-PCR. In contrast to Glimelius *et al.* (1988) and Bjerkvig *et al.* (1989, 1990) they found less collagens and laminin but increased expression of glycosaminoglycans, fibronectin and type IV collagen in 'tumour fragment spheroids' from human gliomas (Paulus *et al.* 1994). However, it has to be kept in mind that tumour fragment spheroids from the same biopsy material may consist of a variable mixture of cells including tumour and stromal elements representing a highly complex coculture system while reaggregation spheroids of defined mixtures of isolated

primary cells are morphologically similar to each other. Tumour fragment spheroids from nonsmall-cell lung cancer, for example, varied from highly cellular spheroids consisting mainly of tumour cells to nearly acellular ones consisting of connective tissue components. Among this heterogeneous population, mixed spheroids with tumour cells located at one end and stromal elements at the other end were observed (Fjellbirkeland *et al.* 1995). This morphological variance is in accordance with a culture system used by Schmittgen *et al.* (1991a,b) mimicking human bladder tumours for pharmacodynamic studies by growing primary human bladder tumour fragments on a collagen gel. Both culture techniques may well represent the essential aspects of an individual tumour while they can hardly be used to study tumour cell biology in general.

Tumour modelling will considerably profit from recent research efforts towards artificial organs using cell lines or isolated cells from primary material. A variety of more or less complex artificial support systems, such as collagen gel matrix (Lazar *et al.* 1995; Nishikawa *et al.* 1996), porous gelatin sponges (Lin *et al.* 1995), a multicompartment capillary system (Gerlach *et al.* 1995), polyurethane foam (Matsushita *et al.* 1991), or simply agarose (e.g. Shiraha *et al.* 1996) have been applied. 3-D-cocultures characterized for histiotypic (re-)organization and organ-specific functions include complex sandwich techniques (i.e. Bader *et al.* 1996) but also spheroids grown on diverse nonadherent surfaces, such as hepatocyte spheroids of human or animal origin (i.e. Nyberg *et al.* 1993; Tong *et al.* 1994; Li *et al.* 1992; Ueno *et al.* 1992; Yagi *et al.* 1993; Yuasa *et al.* 1993; Nemoto *et al.* 1995; Niwa *et al.* 1996; Ota *et al.* 1996; Peshwa *et al.* 1996; Shiraha *et al.* 1996; Wu *et al.* 1996). In general, cocultivation of normal cells, with layers of feeder cells, e.g. nonparenchymal epithelial cells, bovine corneal endothelial cells, or different fibroblasts producing diverse ECM molecules and modulators (e.g. James *et al.* 1992; Jurima-Romet. M *et al.* 1995; Utesch & Oesch 1992) or the initiation of so-called 'hetero-spheroids' consisting of normal organ-specific cells and fibroblasts/nonparenchymal cells (e.g. Endoh *et al.* 1993; Yagi *et al.* 1995) yielded a similar or better organotypic differentiation pattern than spheroid monocultures. Takezawa & associates (1992) developed an interesting coculture method by utilizing a collagen-conjugated thermo-responsive poly N-isopropyl acrylamide polymer as a cell substratum which solidifies above approximately 30°C and dissolves into the medium below this temperature. Human dermal fibroblasts were grown as monolayers on the substratum up to confluence; rat primary hepatocytes were seeded onto the fibroblast monolayer. These hepatocyte-attached fibroblast monolayers were then cultured at temperature below 30°C on nonadhesive substratum and formed hetero- or hybrid-spheroids. In a similar, yet less pretentious attempt with tumour cells, Djordjevic & Lange (1990, 1991); and Lange *et al.* (1992) have used two different culture systems; one was based on packing tumour cells into agglomerates of nonproliferating, but metabolically active, HeLa 'feeder' cells; with the second one defined proportions of HeLa cells were mixed with human adenocarcinoma cells (LoVo) and incubated in bacteriological Petri dishes for 20-45 h to form hybrid-spheroids.

Cocultivation of different tumour cell types or of tumour- with 'feeder' cells in hybrid-spheroids was first undertaken to determine modifications in the radio- or drug-sensitivity in heterogeneous tumours due to the direct cell-to-cell environment, reflecting a major problem in clinical oncology. Djordjevic & Lange (1990, 1991); and Lange *et al.* (1992), for example showed that hybrid spheroids of LoVo and adenocarcinoma cells reflect the intrinsic radio- and chemo-sensitivities of human tumours while Bradley & Pitts (1994) found no evidence for a therapeutically important interaction of the different cell types using a mixed cell system consisting of normal and multidrug-resistant Chinese hamster ovary cells. Tofilon *et al.* (1984, 1987) revealed earlier that BCNU-sensitive rat brain tumour cells became more resistant following mixed culture with resistant rat brain tumour cells in spheroids and Frenzel *et al.* (1995) mixed cisplatin-sensitive and -resistant mouse fibrosarcoma cell subpopulations in 3-D culture in order to analyse how drug-resistant tumour cell fractions alter overall drug sensitivity.

Cocultures of tumour and endothelial cells

Tumour cell-endothelial cell interaction is a relevant cellular target to study the process of tumour cell evasion from blood vessels as well as angiogenesis. While some information of endothelial and tumour cell behaviour could be obtained from a model system of confluent endothelial cells on ECM confronted with tumour spheroids (Knuechel *et al.* 1988; Offner *et al.* 1992), it was found unanimously that the model was not apt for studying angiogenesis. Offner *et al.* (1992) have applied the system to look at the modulation of evasion of tumour cells by oxidative stress and adhesion properties of cells. The production of angiogenic factors can be traced in MCTS and related to the microenvironment of metabolic conditions of cells. This has recently been applied by Waleh *et al.* (1995) who related VEGF production to hypoxia (see also Microenvironment chapter). Antibodies to VEGF as well as a monoclonal antibody to a

hypoxia specific fluorinated bioreductive drug, EF5, were colocalized in sections of HT29 colon carcinoma spheroids (Waleh *et al.* 1995). When the angiogenic process shall be monitored directly two things have to be taken into account. The angiogenic process is partly dependent on the type of endothelial cell (for review see: Folkman 1990), and also depends on its physiological state. Most successful assays to assess angiogenesis in confrontation cultures are using spheroids plus a primary site such as the peritoneal cavity of mice (Zwi *et al.* 1990). Here tumour tissue is vascularized from a physiological environment with capillary sprouts, however, species differences and a semicontrolled environment limit the direct relation of data to the human *in vivo* situation. The concept of Nehls & Drenckhahn (1995) to use endothelial cells, especially microvascular endothelial cells on microcarriers in a fibrin clot shows an experimental setting where sprouting of endothelial cells can be seen and quantified. The quantification is possible since the fibrin matrix is transparent, and different cell types (pericytes, fibroblasts, and smooth muscle cells) could be assessed by growing different cell types on differently stained microbeads. The idea extends the observation of Tsuji & Karasek (1985) who first described tubular formation of microvascular endothelial cells when seeded into collagen I. The authors consider this approach worthwhile to study tumour cell induced angiogenesis and its inhibition under controlled conditions.

There are only few investigations where the presence of blood vessels is considered important in studying invasiveness. For these studies tumour spheroids (Nygaard *et al.* 1995) or tumour cell lines (Booth *et al.* 1997) are confronted with organ cultures and stromal as well as vascular invasion is assessed. It is an interesting finding of these studies as well as in, e.g. bronchial wall biopsies (personal cooperation with Dr Huber, Internal Medicine, University of Munich) that morphologically well preserved endothelial cells line capillaries of stromal cultures for more than 4 weeks in culture. Thus this seems another valid approach of looking at site-specific stromal reaction patterns. The biological variation of primary material, however, has to be taken into account.

Other studies of invasion lead to the topic of the stromal cells other than endothelial cells. The pioneering work and a lot of recent work of invasion in three-dimensional *in vitro* systems has been done with fragments of embryonic chicken hearts as started out by Mareel. Recent work is cited by Mueller-Klieser (1997), and deals with systematic studies on anti-invasive drugs and E-cadherin expression and function (Bracke *et al.* 1993), as well as detailed analysis of cellular mobility and invasion (Hofmann-Wellenhof *et al.* 1995). While invasive properties are not identical with the cellular ability to metastasize, Makiyama *et al.* could show that the invasiveness of mouse sarcoma cells into chicken heart fragments was paralleled with the ability of these cell clones to metastasize (Makiyama *et al.* 1991). Similarly, it was pointed out by Mareel's group that the invasiveness of MCF-7 cells found in their chicken heart model was also found in nude mice and went along with metastasis in the animal model (reviewed in Vermeulen *et al.* 1996).

Cocultures of tumour cells and fibroblasts

The image of scirrhous carcinomas with a clear *desmoplastic reaction* has been described in standard routine pathology for many years and has come into focus again more recently (e.g. Ellis *et al.* 1994; Hewitt *et al.* 1993; Noel *et al.* 1993). Although many solid, epithelial tumours arise without prior detection of fibrosis, and the existence of fibrocytic disease does not necessarily dictate the development of invasive carcinoma (e.g. Rosen 1993), fibroblasts have mainly been investigated as a substrate of tumour cell invasion. Therefore, the phenomenon of enhanced fibroblast proliferation and ECM expression and its role in tumour progression is still controversial (for review: Foidart *et al.* 1994). First data in the literature indicate that not only can the fibroblast influence tumour growth and invasion but *vice versa* tumour tissue may also dramatically affect RNA expression in stromal cells. As an example, it was shown that estrogen-receptor (ER)-negative breast carcinoma cells may induce TGF- (transforming growth factor beta) expression in stromal fibroblasts via the antiestrogen tamoxifen (Butta *et al.* 1992; Van Roozendaal *et al.* 1992)

Bissell's group and associates have tremendously contributed to the understanding of the dynamic interplay between epithelial cells and stromal elements not only in the field of tissue homeostasis in organ development (e.g. Bissell & Hall 1987; Lin & Bissell 1993; Howlett & Bissell 1993; Roskelley *et al.* 1995), but also in breast cancer progression (Weaver *et al.* 1995). They have used a three-dimensional ECM assay, employing a reconstituted basement membrane gel culture model allowing to distinguish normal and malignant breast cells due to their growth and differentiation pattern *in vitro*. Subsequently similar phenotypic differences between normal and malignant primary cells were observed by Bergstraesser & Weitzman (1993) on basement membrane gels. This culture system has been used, for example, to uncover the role of the putative metastasis suppressor gene nm-23 (Barnes *et al.* 1991; Howlett *et al.* 1994; Leone *et al.* 1993). With colorectal

cancers it has recently been documented that the differentiation pattern can be altered by ECM or direct fibroblast-tumour cell contact (e.g. Bosman *et al.* 1993). Accordingly, inoculation of prostate carcinoma cells with prostate or bone marrow fibroblasts into athymic mice resulted in a histodifferentiation of the carcinoma cells. (Gleave *et al.* 1992; Chung 1991, Chung *et al* 1991). In a special hormone-dependent prostate carcinoma, the Dunning tumour, Cunha *et al.* (1991) showed that normal embryonic or neonatal mesenchyme leads to histodifferentiation and loss of tumourigenicity. Similarly, Chung *et al.* (1991) observed a sensitization of prostate carcinoma cells towards androgens affected by interacting fibroblasts and enhanced collagen type I expression in the tumour stroma. While normal mesenchyme inhibited tumour progression of oncogene-initiated epithelial cells in a mouse model (Thompson *et al.* 1993), Hewitt *et al.* (1993) could clearly show that enhanced collagen production of tumour-associated stromal cells in colorectal cancers is not a protective action of host *vs.* tumour spread since it is absent at the invasion front. This is in accordance with the observation by Lefebvre *et al.* (1995), who described a significant proliferation thrust of breast adenocarcinoma cell lines MCF-7 and BT-20 cocultered with fibroblasts derived from breast tumour biopsies in a microchamber slide system. Similarly, Dong-Le Bourhis *et al.* (1997) observed an inhibition of MCF-7 cell growth by normal breast fibroblasts but not by fibroblasts from breast tumour biopsies in a dual chamber culture system; fibroblast-conditioned media were ineffective. In contrast, Hofland *et al.* (1995) documented a mitogenic effect of medium conditioned by tumour-associated fibroblasts on primary human breast cancer cells.

The fact that fibroblasts may produce angiogenic factors such as VEGF *in vitro* under hypoxic stress (Hlatky *et al.* 1994) also raises the important question to what extent stromal fibroblasts contribute to tumour vascularization. In fact, using an *in vitro* coculture model of microvessels with fibroblasts in a three-dimensional collagen gel, Villaschi & Nicosia (1994) could show that fibroblasts stabilized the microvascular network and prolonged its lifespan *in vitro* associated with increased deposition of subendothelial ECM. In parallel, fibroblast conditioned medium stimulated angiogenesis in the rat aorta assay.

Most of the *in vitro* data mentioned, have been carried out in monolayer coculture, ECM gel culture systems, or using fibroblast-conditioned medium. However, the establishment of new, stroma cell-mediated therapeutic concepts requires simultaneous observations on ECM-tumour cell, fibroblast-tumour cell, and auto-, juxta-, and paracrine interactions. Although the spheroid model is a useful tool in the study of three-dimensional, heterologous cell-cell interactions in developmental biology, it has rarely been used for similar questions on the phenomenon of *desmoplasia* and on the influence of stromal fibroblasts on the differentiation of tumour cells. Brouty-Boyé (1994) for example showed that cocultivation of breast MCF-7 MCTS with fibroblast monolayers resulted in a progressive histological re-differentiation of the tumour cells associated with increased mucin production. In order to investigate the complex reciprocal fibroblast-epithelial interactions in a well-defined microenvironment, we have established a heterologous spheroid coculture system of human bladder cancer cells and fibroblasts using two differently differentiated bladder carcinoma cell lines RT4 (highly differentiated) and J82 (nondifferentiated, highly aggressive) and two different fibroblast cell lines of normal human origin N1 (adult skin) and WI38 (foetal lung) (Schuster *et al.* 1994). Cocultivation of aggregates was initiated following a 4-5 day interval of spheroid monoculturing. Tumour cells were characterized by 3-D proliferation while the fibroblast aggregates did not grow. Immunohistochemistry revealed that interactions varied with tumour and fibroblast cell type with N1/RT4 and N1/J82 spheroid cocultures reflecting the *in vivo* situation and invasive properties of the tumour cells. Our results on extracellular matrix composition, proliferation, differentiation, and invasion within RT4/N1 spheroid cocultures were recently confirmed by Booth *et al.* (1997) who investigated stromal and vascular invasion of a variety of human tumour cell lines including RT4 in 'composite organ culture'. In this novel type of coculture system, bladder wall connective tissue was isolated from fresh samples, and used as 0.5 cm^2 (surface) pieces for coculture with different bladder carcinoma cell lines to form so-called 'organoids'.

Based on our experience with 3-D bladder cancer/fibroblast cocultures we have recently tried to partly simulate scirrhous breast carcinomas *in vitro* by cocultivation of different breast carcinoma spheroid types such as BT474, MCF-7, ZR75-1, and T47D with N1 fibroblast aggregates. All of the breast carcinoma spheroid types tested so far connect and may overgrow the nonproliferating N1 aggregates while mixing of the cells similar to that observed in J82/N1 cocultures could not be detected (Figure 3a,b). An intense positive immunohistochemical staining of the ECM could be shown in N1 aggregates in coculture for fibronectin and collagen type I (Figure 3c,d) whereas laminin was poorly positive and collagen type IV was negative. Immunostaining on BT474/N1 cocultures also implies that fibronectin expression might be slightly induced in BT474 cells close to the rim of the fibroblast aggregates decreasing

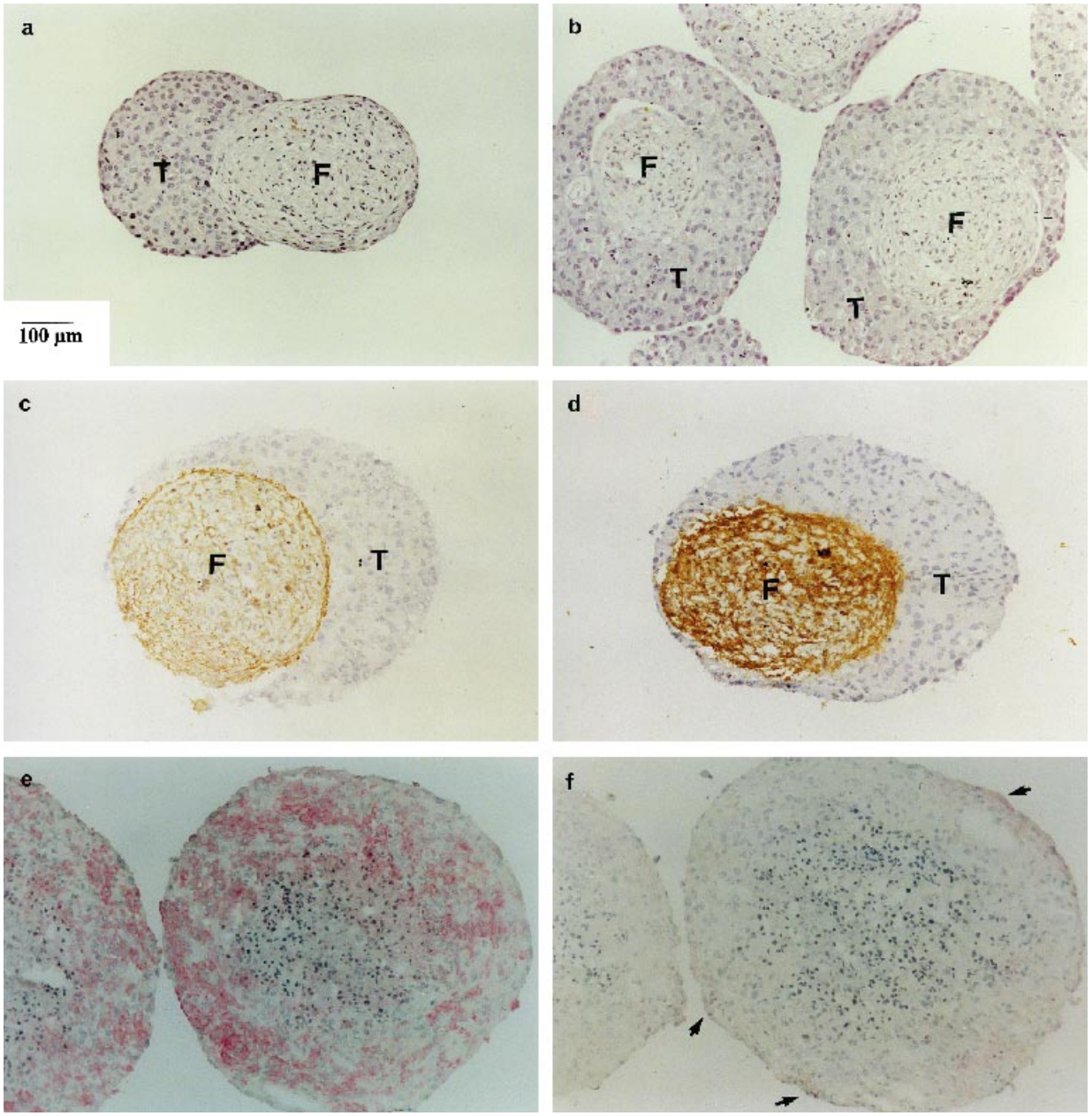


Figure 3. a & b, H & E stained, 5 μm sections of paraffin-embedded BT474/N1 spheroid cocultures (human breast carcinoma/fibroblast) after 2 (a) and after 8 (b) days in coculture. The initial size of spheroids prior to cocultivation was 300-400 μm for BT474 MCTS and 250-350 μm for N1 aggregates. c & d, Immunohistochemical staining of ECM components fibronectin (c) and collagen I (d) in 5 μm frozen sections of BT474/N1 cocultures after 8 days in coculture using a peroxidase technique and DAB (diaminobenzidine) for detection. e & f, Immunohistochemical staining for monocyte/macrophage markers CD11c (e) and HLA-DQ (f) in 5 μm frozen sections of J82 human bladder carcinoma spheroids infiltrated by elutriation-purified primary monocytes using the APAAP method and Fast Red for detection; counterstain with hematoxilin. F, fibroblasts; T, tumour cells; Bar = 100 μm applies to all parts a-f.

with distance to the fibroblast cells, a finding we currently try to confirm by Northern-blotting. Staining for the proliferation-associated antigen Ki-67 (Mib-1 antibody) indicated that the breast carcinoma cells are not capable of inducing fibroblast proliferation in this *in vitro* system. Also, it should be emphasized that N1 fibroblast aggregates both, in 3-D mono- and coculture, are negative for α-smooth muscle actin using conventional immunohistological techniques while a considerable percentage of cells is positive in monolayer culture even at cell confluence. Several investigators have identified 'myofibroblasts' as an important subpopulation of stromal fibroblasts in diverse reactive lesions including scirrhous breast carcinomas (e.g. Rønnov-Jessen *et al.* 1992, 1995; Chiavegato *et al.* 1995; Powell *et al.* 1995; Raju *et al.* 1990; Brouty-Boyé *et al.* 1991; Gabbiani 1996). Rønnov-Jessen *et al.* (1992) described few or no α-smooth muscle actin immunoreactive cells in primary fibroblast cultures from normal breast tissue but high frequencies of about 70% in fibroblasts isolated from breast carcinomas. Recently, Martin *et al.* (1996) showed very clearly that there is evidence for myofibroblasts being involved in invasion and metastasis of colourectal tumour cells. Data indicate that the type of fibroblast is critical in designing the tumour fibroblast mode. Some investigators preferentially apply foetal or embryonic fibroblasts that may to some extent reflect tumour-fibroblast phenotype (e.g. Colletta *et al.* 1990; Haggie *et al.* 1987). In addition to establishing primary fibroblasts from concomitant tissues, we and others are currently studying paracrine influences that may affect myofibroblast differentiation. Factors such as TGF-β that have been described to induce α-smooth muscle actin expression in rat fibroblast monolayer cultures (Rønnov-Jessen & Petersen 1993) require detailed investigation in well-defined 3-D coculture systems.

Future research will also need to take into account that different stromal cells may have synergistic/additive effects. An interesting study by Janusz & Hare (1993) showed for example that fibroblasts and macrophages of human and mouse origin in coculture incubated on heat-killed cartilage disks resulted in disk degradation and release of proteoglycans while macrophages alone were less effective and fibroblasts did not affect the cartilage. Cartilage degradation was blocked by diverse metalloproteinase inhibitors indicating that the release of extracellular matrix degrading enzymes may be an interactive process between macrophages and fibroblasts.

Cocultures of tumour and immunocompetent cells

Interactions between cells of the immune system and tumour cells are ambivalent and still poorly understood. Immune cells are capable of recognizing and destroying malignant cells, but may also induce tumour growth and promote angiogenesis by secreting growth factors and diverse biological response modifiers. Cells involved in the immune response to malignancy are mainly cytotoxic T-lymphocytes, NK-cells (natural killer cells), and monocytes/macrophages. While nonspecific tumour-cell recognition was described for NK-cells and monocytes/macrophages, cell-mediated immunity to tumours by T-cells is a specific immune response to various tumour antigens in combination with MHC molecules (major histocompatibility). Also, the mechanism of tumour cell destruction varies among the different cell populations. T-lymphocyte and NK-cell activity seem to depend on direct cell-to-cell contact whereas macrophages may also act via soluble mediators (Bonta & Ben-Efraim 1993; Lanier & Phillips 1992).

The majority of *in vitro* studies on immune-cell mediated tumour-cytotoxicity published so far utilizes monolayer cultures or single cell suspensions of tumour cells as target. In addition, a Transwell culture system with upper and lower compartment separated by a polycarbonate-treated membrane may be useful to investigate cytokine mediated effects of specific cell types such as PBMs (peripheral blood mononuclear cells) (e.g. Neuber *et al.* 1995). However, if one is looking for cell-contact dependent mechanisms these culture techniques hardly reflect the *in vivo* situation since immune cells must have the capacity to infiltrate the tumour tissue prior to cell destruction. As a result, several investigators have recently applied multicellular tumour spheroids as a model system to analyse migration and cytostatic or cytotoxic activity of immune cells. A relatively new approach to eliminate tumour cells in human malignancies is an adoptive immunotherapy utilizing lymphokine-activated killer (LAK) cells. Iwasaki *et al.* (1990) described the infiltrative and cytolytic activity of interleukin (IL)-2 stimulated peripheral blood lymphocytes/LAK cells in a human glioma spheroid model. They found that LAK cells infiltrate the MCTS and destroy the glioma cells, whereas control cells (unstimulated blood lymphocytes) showed no effect. Phenotypical and morphological analyses of the infiltrating LAK cells revealed a mixed population consisting of CD3-positive T-cells and CD16-positive NK cells (Iwasaki *et al.* 1990). Accordingly, Jääskeläinen *et al.* (1989) compared single cell targets with glioma MCTS cultures and could show that ^{51}Cr release was clearly delayed in spheroids as compared to the corresponding single cell suspensions indicating the relevance of cell infiltration as an additional factor influencing LAK cell kill. In addition, infiltration and

cell destruction by LAK cells in 3-D culture seemed to depend on the tumour cell type. The authors conclude that the LAK-MCTS cell system may well be used to discriminate between resistant and susceptible tumour cell fractions for an adoptive immunotherapeutic treatment (Jääskeläinen *et al.* 1989). They also could show that the formation of cytoplasmic blebs, a characteristic effect of T-cells, natural killer cells, and LAK cells on single target cells, occurred in the LAK-glioma spheroid model but was not limited to the effector-target contact area suggesting that LAK cells release membrane-damaging agents that remain active in the spheroid micromilieu (Jääskeläinen *et al.* 1990). In a similar study, Kammerer & Kleist (1995) demonstrated that LAK cells were not capable of infiltrating a colorectal carcinoma spheroid type. They argued that infiltration of LAK cells may depend on an 'intact' tumour tissue architecture involving tumour stroma and the development of carcinoembryonic antigen which is mainly expressed on the apical membrane of the tumour cells. However, this hypothesis needs to be proven in the near future, in particular since Pervez *et al.* (1989) clearly showed that spheroid cells may drastically differ in terms of polarity using two large-bowel-derived adenocarcinoma cell lines.

Data on immune cell migration using MCTS were already published in 1987 by Wilson and Lord who injected MCTS in mice and analysed infiltration and cytolytic capacity of host cells *in vivo/ex vivo*. Separation of MCTS infiltrated with cytolytic cells *in vivo* revealed that two cytolytic cell populations were present, with macrophages infiltrating the MCTS independent of immunization, and T-lymphocytes being present only in mice immunized with the corresponding tumour cells. NK-cells could not be detected (Wilson & Lord 1987). Thomsen *et al.* (1992) showed that stimulation of host-cell infiltrated EMT-6 mouse mammary carcinoma spheroids by flavone-8-acetic acid and 5,6-dimethylxanthenone-4-acetic acid activated the nitric oxide synthesis in tumour-cell associated macrophages and thereby led to tumour cell destruction. Production/release of nitric oxide as a result of direct macrophage-tumour cell interaction has also been demonstrated in human MCTS (Konur *et al.* 1996a; Zembala *et al.* 1994). However, in contrast to the murine system, it is still unclear if human monocytes/macrophages or the tumour cells themselves are the major source of nitric oxide.

It has been known for some time, that macrophages (MACs) are a major component of the leucocyte infiltrate in many tumours. Yet, the relationship between these tumour-associated macrophages (TAMs) and tumour cells is ambivalent. TAMs may destroy tumour cells *in vitro and in vivo* but may also support tumour development, metastasis and angiogenesis (Mantovani *et al.* 1992). In order to investigate the effect of the tumour microenvironment on activation and differentiation of human monocytes (MOs) and macrophages (MACs) we and others have established an experimental system by cocultering human MO/MAC with MCTS. MOs and MO-derived MACs infiltrated MCTS. Figure 3(e,f) represents J82 human bladder carcinoma spheroids cocultured with freshly isolated primary human MOs for a period of 7 days. Immunohistochemical staining was performed using monoclonal antibodies for CD11c, a common MO/MAC marker, and for HLA-DQ. MO infiltrated the entire MCTS as shown by CD11c immunoreactivity in Figure 3(e), whereas staining for HLA-DQ occurred in the spheroid periphery only (Figure 3f).

Audran *et al.* (1994) have shown that infiltration of MO-derived MACs into MCTS was inhibited by anti-ICAM (CD54) antibody. They reported a cytostatic effect of infiltrating MACs on tumour cells resulting in a proliferation arrest in MCTS but no cytolytic activity. Tumour cytostasis was not related to TNF-alpha.

Infiltration of MACs into MCTS also seems to be dependent on the type of MAC. Hauptmann *et al.* (1993) described inflammatory MACs localized at the periphery of MCTS and resident MACs that were predominantly found in central tumour regions. These different types of MACs showed a controversial behaviour in terms of cytostatic activity: inflammatory macrophages inhibited while resident macrophages stimulated tumour cell proliferation (Hauptmann *et al.* 1993).

MOs, the circulating precursor of MACs in the blood stream, migrate into the body's cavities and tissues where they undergo MAC differentiation modulated by the specific microenvironment. It is also under discussion that proliferation of tissue macrophages may play a role in preserving the balance of tissue MACs. Most likely, TAMs are originated from MOs that have infiltrated tumour tissue. Thus, we investigated migration and differentiation of freshly isolated blood MOs in a human bladder MCTS model and found that MO and MO-derived MAC infiltrate and spread throughout the entire MCTS within 24 h. The infiltration/migration seems to be specifically regulated by tumour cell factors since fibroblast aggregates were not infiltrated by MOs/MACs. Further cultivation of MOs in the tumour microenvironment inhibited the expression of maturation-associated MAC antigens. TAMs cultured in MCTS of the undifferentiated bladder carcinoma cell line J82 showed no expression of carboxypeptidase M, MAX.3 and CD51, whereas TAMs had a normal maturation-associated

phenotype in nontumourigenic HCV29 MCTS. Beside antigen expression we have analysed the cytokine response of TAMs in J82 MCTS. TAM secreted high amounts of IL-1β and IL-6 but low amounts of TNF-α as compared to control MACs differentiated without tumour contact. This cytokine pattern is typical for MOs, also indicating a disturbed differentiation of MOs into MACs in the tumour environment (Konur *et al.* 1996b). Since cytotoxicity of MACs is dependent on differentiation, i.e. cytotoxicity is higher in mature MACs than in MOs (Andreesen *et al.* 1983), the tumour seems to suppress the differentiation process resulting in a self-protection against MAC cytotoxicity. Alterations in the differentiation pattern of MO/MAC and reduced cytotoxicity in cancer has also been reported by others (e.g. Krishnan *et al.* 1980; Siziopikou *et al.* 1991). A similar inhibition of MO/MAC-differentiation could not be detected in a monolayer coculture system with tumour cells supporting the hypothesis that MCTS/MO cocultures better reflect the *in vivo* situation and may well be applied to test new attempts of adoptive tumour therapies utilizing immune cells such as MAC.

Vaccination with dendritic cells is a new approach in tumour therapy. Therefore we are currently investigating the interaction of dendritic cells with tumour cells in a MCTS system. Preliminary experiments show that dendritic cells derived either from CD34 positive progenitor cells cultured with GM-CSF, TNF-α and stem cell factor, or dendritic cells derived from monocytes differentiated in the presence of IL-4 and GM-CSF infiltrate MCTS. After 20 hours dendritic cells could be detected throughout the MCTS (unpublished data). This model system for the interaction of tumour cells with dendritic cells may be of particular interest with regard to tumour therapy utilizing dendritic cells.

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