Pergamon

European Journal of Cancer Vol. 31A, Nos 13/14, pp. 2324–2329, 1995

0959-8049/95 $9.50+0.00

0959-8049(95)00439-4

Original Paper

The Influence of the Local Environment on Tissue Architecture of Colorectal Carcinoma (CRC) Cell Aggregates and its Consequence for Tumour Attack by Lymphocytes *In Vitro* and *In Vivo*

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We analysed colorectal carcinoma (CRC) specimens, tumour cell spheroids and artificial tumours (ArTs) for tissue architecture, carcinoembryonic antigen (CEA) expression and lymphocyte infiltration. Two distinct organisation forms of well-differentiated CRC cells were found *in vivo* and *in vitro*. Tumour cells having contact with the tumour stroma in primary tumours, and tumour cells growing within a stroma-like structure *in vitro* (ArTs) were arranged as pseudoglands. In contrast, tumour cells grown as spheroids or tumour cells having lost contact with the tumour stroma in primary tumours, and most probably in the circulation, showed an inversion of the architecture of these pseudoglands, presenting their apical cell membrane to the environment. These different tumour cell formations affect lymphocytes attacking the tumour, which need contact with specific cellular membranes of polarised tumour cells, depending on the tumour architecture. Recently, we demonstrated that the CEA expression of CRC cells correlated with their resistance against LAK-cell lysis. Since CEA is mainly expressed on the apical membrane of the tumour cells, independent of the tissue architecture, the change from the pseudoglandular to the spheroid-like formation may represent an escape mechanism for malignant cells.

Key words: spheroids, ArTs, tissue architecture, human tumour cells, LAK-cells
Eur J Cancer, Vol. 31A, Nos 13/14, pp. 2324–2329, 1995

INTRODUCTION

THE NATURE of lymphocyte/tumour cell interaction is one of the basic questions in tumour immunology. *In vitro* studies carried out on this interaction often ignore the *in vivo* situation. For instance, the most commonly used method to identify cytotoxic activity of lymphocytes against tumour cells is the chromium release assay using single cells as targets, but *in vivo* few tumours exist in this form, and indeed most are solid. Therefore, we and other investigators have recently used the three-dimensional spheroid model to simulate more realistically *in vitro*, the *in vivo* situation [1–3]. For polarised tumour cells, such as differentiated colon carcinoma cells, the three-dimensional architecture is particularly important, because polarised epithelial cells comprise an apical, basal and lateral membrane domain which all have distinct functions and differ in their protein and lipid components. For example, glycosyl-phosphatidylinositol (GPI) anchored molecules such as carcinoembryonic antigen (CEA), are preferentially expressed on the apical area of the cell membrane [4]. In contrast, molecules which are important for the adhesion of intra-epithelial lymphocytes are found to be expressed mainly on basolateral membranes [5]. Perhaps it is important for the result of a lymphocyte/tumour cell interaction, which area of the cell membrane is attacked by lymphocytes. Since the possible attacking site is most certainly determined by the tumour cell architecture, the aim of our study was to investigate which culture conditions influence the organisation of polarised tumour cells and the consequence a given tissue structure may have for tumour attack by lymphocytes *in vitro* and *in vivo*.

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Received 15 May 1995; accepted 16 May 1995.

MATERIALS AND METHODS

Tumour cell cultures

Human tumour cell cultures were performed as described previously [3]. In brief, colorectal carcinoma (CRC) tissue samples (n = 6) were obtained surgically. After washing the tissue extensively, it was aseptically cut into pieces of approximately 1 mm^3, which were placed in 250 ml flasks (Greiner, Frickenhausen, F.R.G.) and cultured until epithelial cells and fibroblasts migrated from the explants. Separation of epithelial

cells and fibroblasts was achieved by selective passaging with 1000 U/ml collagenase (Biochrom, Berlin, F.R.G.). During dissociation, the flasks were monitored under an inverted microscope (Olympus Optical Co., Ltd, Japan) and digestion was stopped when epithelial cells, but not fibroblasts were detached. This procedure was repeated weekly until all fibroblasts were eliminated from the tumour cell cultures. Pieces of tumour stroma were digested with 1000 U/ml collagenase to obtain a single cell suspension of fibroblasts, which was then expanded to a monolayer and routinely passaged by mild trypsinisation. After six passages, no epithelial tumour cells were detectable in these cultures.

Culture media and culture conditions

Tumour cells and fibroblasts of the tumour stroma were cultured in Dulbecco's modified Eagles' medium Nut Mix/F12 (1:1) supplemented with 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, 10 mM Hepes buffer, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Paisley, U.K.). This medium is hereafter referred to as tumour medium (TM). Lymphocytes were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated human AB serum (Sigma, Deisenhofen, F.R.G.), 2 mM L-glutamine, 10 mM Hepes buffer, 100 U/ml penicillin and 100 μg/ml streptomycin, hereafter referred to as lymphocyte medium (LM). Incubations and cultures were performed at 37°C in a 5% CO_2 humidified incubator (Heraeus, Hanau, F.R.G.).

Production of homotypic spheroids

Spheroids were grown in six-well culture plates (Costar, Cambridge, Massachusetts, U.S.A.) on a thin layer of agar (0.5 w/v % agar (Difco, Detroit, Michigan, U.S.A.) in distilled water) to avoid cell attachment. Only half the TM was changed twice a week. The size of the spheroids was measured using an inverted microscope supplied with a microscale (Olympus, Hamburg, F.R.G.).

Production of artificial tumours (ArTS)

ArTs comprising CRC cells, colon fibroblasts and—as a supportive skeleton—fibrous tissues prepared from human colonic mucosa were established as described previously [6]. In brief, to prepare the fibrous tissue, normal human colon tissue was aseptically cut into pieces of approximately 1 cm in diameter before digestion with trypsin/EDTA (0.05/0.02%) solution (Seromed, Berlin, F.R.G.) for 10 days. Each day the pieces were rinsed in phosphate-buffered saline (PBS) and the trypsin/EDTA solution was renewed. Thereafter the pieces were rinsed in PBS (Seromed) and incubated in TM containing 100 U/ml hyaluronidase and 100 U/ml deoxyribonuclease I (Serva, Heidelberg, F.R.G.) for 4 days. After washing to remove the enzymes, the samples were stored in PBS in an incubator (at 37°C) until use. After pretreatment of the fibrous tissue with collagenase, tumour cells and fibroblasts were detached by treatment with 500 U/ml collagenase, then plated on the fibrous tissue by sedimentation in a long test tube. Cells were allowed to grow on the fibrous tissue in this tube overnight. The resulting aggregate was then transferred into a 25 ml culture flask, which was gently shaken every day to avoid attachment to the culture substrate.

Determination of antigen expression

To confirm the nature of the cultured cells, immunoperoxidase staining for CEA and for expression of intermediate filaments was performed on slides of formalin fixed, paraffin-embedded tumour samples and three-dimensional models or on cultured cells (chamber slides) using anti-CEA, anti-vimentin and anti-cytokeratin monoclonal antibodies (MAbs) (Dako, Hamburg, F.R.G.) and the standard avidin biotin technique.

Production of rIL-2 activated lymphocytes

Peripheral blood mononuclear cells (PBMC) were obtained from heparinised blood of healthy donors. PBMC were separated by centrifugation on a Ficoll/Hypaque (Pharmacia, Uppsala, Sweden) density gradient (density = 1.077 g/ml). Cells from the interface of the gradient were harvested and washed twice with RPMI-1640 medium. Then the lymphocytes were cultured in LM supplemented with 100 U/ml recombinant interleukin-2 (rIL-2) for 7 days before they were used for the experiments.

Detection of infiltrating lymphocytes in tumours and three-dimensional tumour models

Spheroids or the ArTs were incubated with 5×10^6 rIL-2 activated lymphocytes for 8, 24 or 48 h. After formalin fixation and paraffin embedding of the three-dimensional models with the lymphocytes, sections were stained for the leucocyte common antigen using monoclonal antibodies (Dako, Hamburg, F.R.G.) and the standard avidin biotin technique to visualise penetrated lymphocytes in the three-dimensional models. Tumour infiltrating lymphocytes (TIL) in the tumour specimens were stained similarly.

RESULTS

Growth, morphology and CEA expression of CRC cells grown as spheroids

Well differentiated CRC cells plated on agar formed small aggregates within 24 h, which usually grew very slowly, reaching a size of 200 μm in diameter after 14 days. Sections of these spheroids showed polarised cell arrangements (Figure 1d). Electron microscopy performed on spheroids confirmed that the cells were well polarised, with microvilli present only at the apical surface of the tumour cells at the edge of the spheroids in contact with the culture medium (Figure 2c). Upon further culture, these aggregates grew, but the centre of the spheroids became necrotic. The space between the surface cell layer and the necrotic area was filled by unpolarised cells. However, during culture, the spheroids were separated from the culture medium by the well polarised surface cell layer. The tumour cells of this layer were connected by tight junctions which provided the barrier function of epithelia to the environment, in this case, the culture medium. Immunostaining against CEA showed the most intensive staining on the apical membrane of the polarised cells, which is normal for GPI anchored proteins (Figure 3a), resulting in the highest CEA concentration on the surface of the spheroids. The unpolarised cells were stained more evenly with strong staining of the intercellular spaces.

Growth, morphology and CEA expression of CRC cells grown in ArTs

The fibrous tissue and the cells formed a stable aggregate within 4 days of culture. The well differentiated CRC cells had contact with the artificial tumour stroma via their basal membrane. Within the ArTs, glandular-like structures formed consisting of lumina surrounded by closely apposed cells with basally orientated nuclei (Figure 1b), which was similar to the structure observed in primary tumours (Figure 1a) but inverted when compared to the arrangement of the cells in spheroids

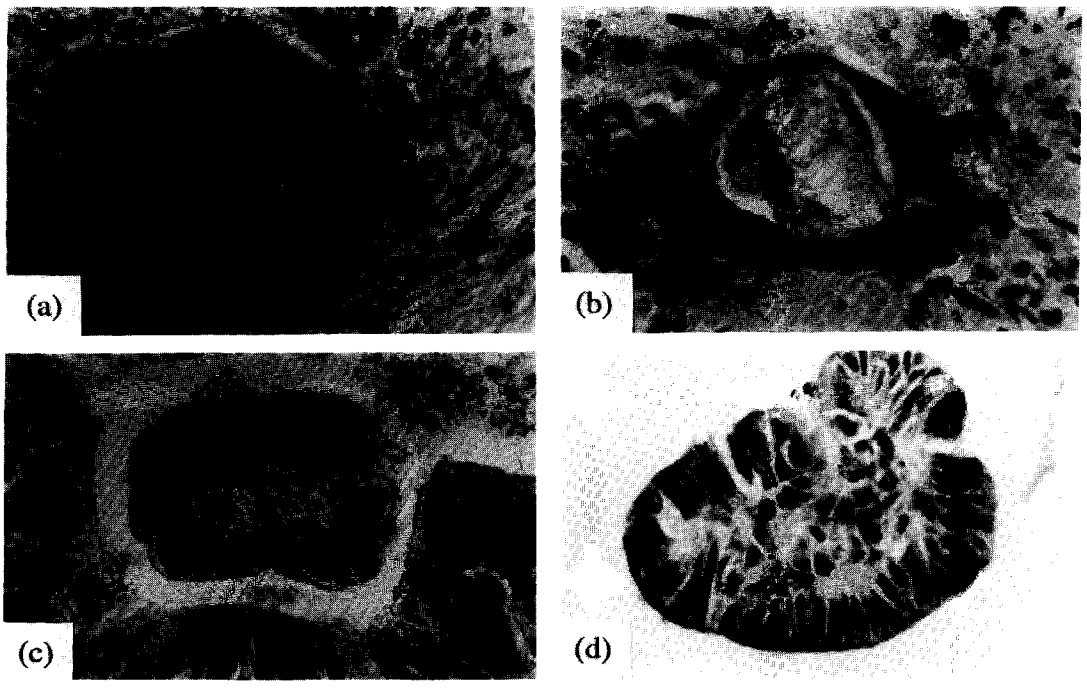

Figure 1. Tumour cell architecture *in vivo* (a, c) and their *in vitro* counterparts (b, d). (a) Original patient tumour forming pseudoglandular tumour cell cords supported by the intratumoral stroma. (b) ArT grown *in vitro*. Note the preservation of the pseudoglandular structures and the orientation of the basal cell membrane to the stroma-like structure of the ArT. (c) Tumour cells of the primary tumour without any contact to the stroma showing inverted architecture of the pseudoglands. (d) CRC cells grown *in vitro* as multicellular spheroids displaying carcinoma cells with epithelial characteristics especially on the surface cell layer with the orientation of their apical membranes to the external border of the spheroid.

(Figure 1d). The surface of tumour cell aggregates in the ArT consisted of basal cell membranes. When tumour cells grew on the surface of the ArTs, their basal membrane was always orientated towards the stroma and the apical membrane to the culture medium. CEA was mainly expressed on the apical membrane resulting in its secretion into the lumen or into the culture medium. CEA staining on the tumour cell/stroma interface was generally very weak.

Tumour cell architecture and CEA expression in CRCs

Tumour cells which were in contact with the stroma usually formed pseudoglandular structures (Figure 1a) showing the most intensive staining for CEA in the lumina (Figure 3b). Those tumour cells which had no contact with the stroma formed aggregates with a spheroid-like architecture (Figure 1c) and CEA expression. Thus the surface of tumour cell islands having had contact with the tumour stroma was formed by the basal cell membranes, while the surface of cell aggregates having had no contact with the stroma was made up of apical membranes.

Lymphocyte infiltration into homotypic spheroids

Paraffin sections of spheroids, obtained from three different CRC cell lines which were incubated with lymphokine-activated killer (LAK)-cells for 24 and 48 h were stained for CD45 to detect the amount and depth of LAK-cell infiltration. Interestingly, the spheroids preserved their integrity throughout the time of coculture. In addition, no change in the density of the spheroids was observed. After 24 h, no infiltrating LAK cells were found within the spheroids, and only a few LAK cells adhered to the spheroid surface (Figure 3a). The histological structure of LAK-cell-treated spheroids was highly preserved with no damage visible on the viable rim, while the central area was similarly necrotic in treated and control spheroids. After 48 h, some LAK-cells had usually infiltrated the outer cell layers, but a dense infiltration never occurred. Both the control spheroids and the spheroids cocultured with LAK-cells lost their integrity if cultured for longer than 3 days, obviously due to the non-optimal culture conditions. Overall, these results demonstrated the high resistance of the spheroids against LAK-cell adhesion and infiltration. In contrast, fibroblast spheroids became infiltrated and lost their integrity within 24 h of coculture, and severe spheroid destruction occurred after 48 h rendering staining no longer possible.

Lymphocyte infiltration into the ArTs

ArTs were infiltrated by LAK-cells via the collagen fibrous tissue and the fibroblasts, thereby reaching the epithelial tumour cells from their basal membrane (Figure 2b). After only 8 h, a strong LAK-cell infiltration could be observed in every part of the tumour model, except in dense cell aggregates made up of fibroblasts and tumour cells. However, a few lymphocytes were always noted in close contact with the basal membrane of polarised tumour cells, a finding similar to the situation of the original tumour *in vivo* (Figure 2a). Following further coculturing, the ArTs lost their density, possibly due to the damage of the fibroblasts, which were responsible for the contraction of the ArTs. After 24 h, ArTs showed a dense LAK-cell infiltration, but an accumulation of lymphocytes around the

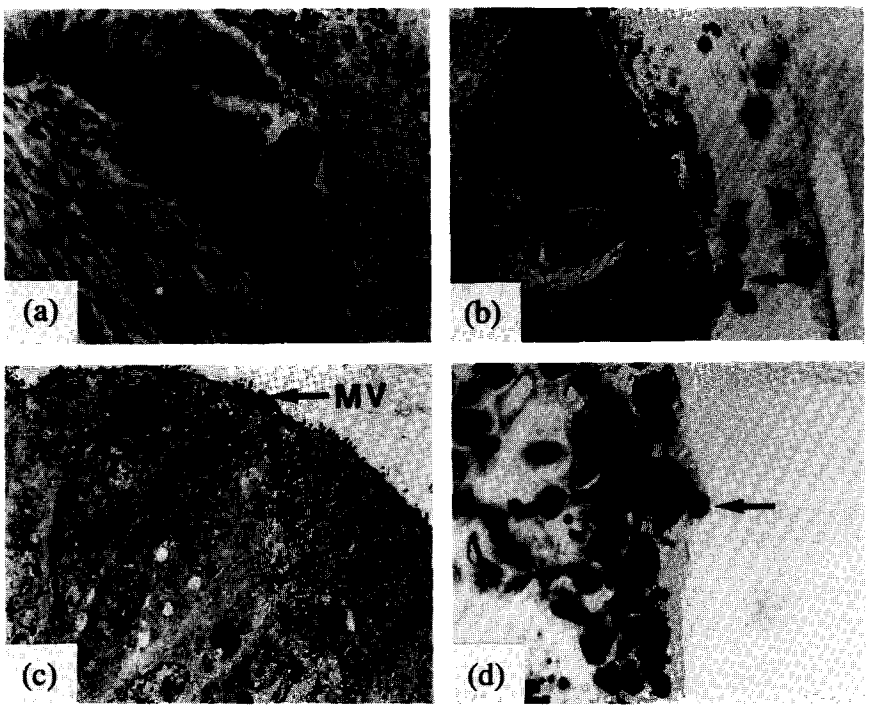

Figure 2. (a) Original patient tumour with lymphocytes (marked by the arrows) reaching the tumour cells via the tumour stroma at their basal membrane. (b) ArT grown *in vitro* infiltrated by lymphokine-activated killer (LAK)-cells (marked by the arrows) attacking the tumour cells at their basal membrane similar to the situation in the original tumour. (c) Transmission electron micrograph of the peripheral rim of a CRC cell spheroid showing the apical membrane of the cells with microvilli (MV) and tight junctions (TJ) between the cells, as the only possible attacking site for lymphocytes *in vitro*. (d) LAK-cell adhering on the surface of a spheroid and therefore on the apical membrane domain of a polarised CRC cell.

tumour cell islands, suggesting that a specific recognition of the tumour cells could not be demonstrated. Furthermore, nearly all LAK-cells were seen in the stroma-simulating part of the ArTs, while the tumour cell islands were almost free of LAK-cells. After 48 h, most of the ArTs were damaged, with only a few viable tumour cell aggregates and no viable fibroblasts found.

Tumour infiltrating lymphocytes (TIL) in primary tumours

Tumour infiltrating lymphocytes were, in general, restricted to the periphery of malignant tumour islands adjacent to the vascularised stroma. Lymphocytes having contact with the tumour cells were located on the external surface of the basement membrane, if it existed, at the basolateral surface of the tumour cells (Figure 2a).

DISCUSSION

In this report, we have demonstrated the possibility of obtaining the desired tumour cell architecture *in vitro* by using different culture substrates. For instance, tumour cells having a suitable extracellular matrix (ECM) filled with fibroblasts, will adhere to them with their basal cell membrane, developing an apical cell membrane opposite, leading to pseudoglandular formations with lumina. If the same tumour cells are grown in culture medium only, they will organise in a way which results in an inverse architecture of these pseudoglands. Interestingly, in primary tumours, polarised tumour cells having lost the contact with the tumour stroma form aggregates that show a spheroid-like architecture. This might indicate that tumour cells in the peritoneal cavity or travelling in blood or lymph vessels to distant organs, most likely form spheroid-like aggregates since they have an environment without any stroma. If we consider that the natural function of epithelial cells is to separate the body from the environment with their apical membrane [7], the change from pseudoglandular to spheroid-like architecture seems to be an adaptation to the mechanical stress tumour cells are exposed to in the circulation. When tumour cells reach the site of metastases formation and are arrested in the capillary bed, they have to reorganise their structure—when they infiltrate the target organs, they form pseudoglandular structures [8]. We conclude that the organisation of well differentiated CRC cells depends on their environment *in vivo* and *in vitro*. The different organisation of epithelial tumour cell aggregates in different compartments of the human body and their *in vitro* similarities are schematically shown in Figure 4.

The fundamental differences of *in vitro* and *in vivo* results are the reason for many problems in the search for potent cancer therapies, caused, at least in part, by the uncritical interpretation of the results of *in vitro* tumour models. The fact, for instance, that polarised tumour cells showed *in vitro* two different organisation forms clearly demonstrates that we have to improve our models to obtain results pertinent to the *in vivo* situation. Since the tumour cell arrangement *in vivo* can be simulated more closely by using spheroids and ArTs, we now have the possibility of investigating *in vitro*, the effect of cancer therapies on tumour cells in distinct parts of the human body. The vector function of epithelial cells seems to be important with regard to nearly all cytotoxic chemotherapies. For instance, anticancer drug uptake seemingly depends on tumour cell architecture, because P-glycoprotein, which functions as an efflux pump for chemotherapeutic components in most colon cancer cells, is preferentially expressed on the apical part of the cell membrane [9–11]. Furthermore, it has been shown that the expression of antigens used for antibody immunotargeting differs dramatically between

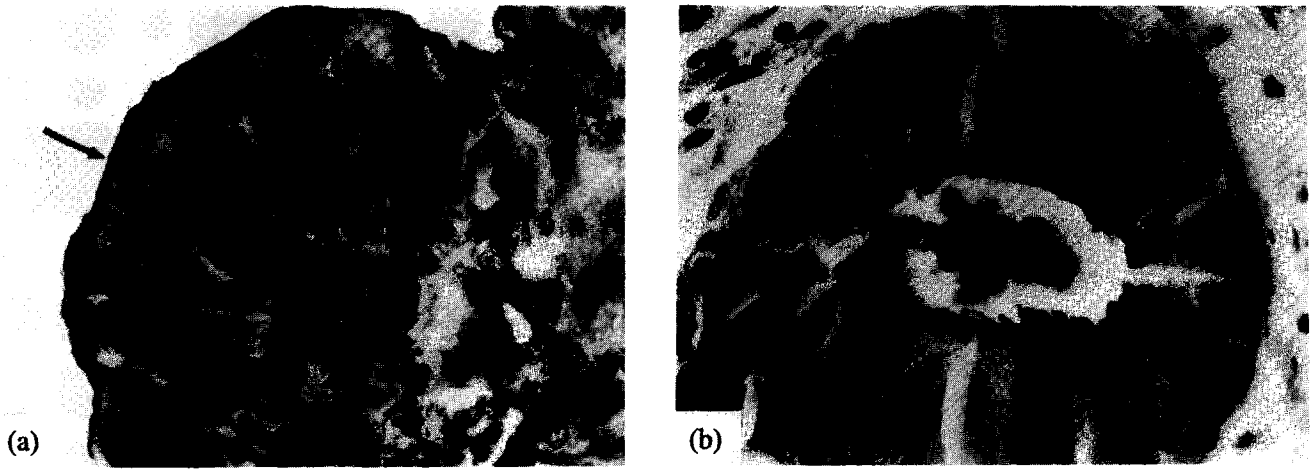

Figure 3. Immunoperoxidase staining of CRC cells with an anti-CEA antibody. (a) Spheroid-like tumour cell formation with high expression of CEA on the surface of the tumour cell aggregate. (b) Pseudoglandular formation with high expression of CEA in the lumen and low expression on the surface of the tumour cell aggregate.

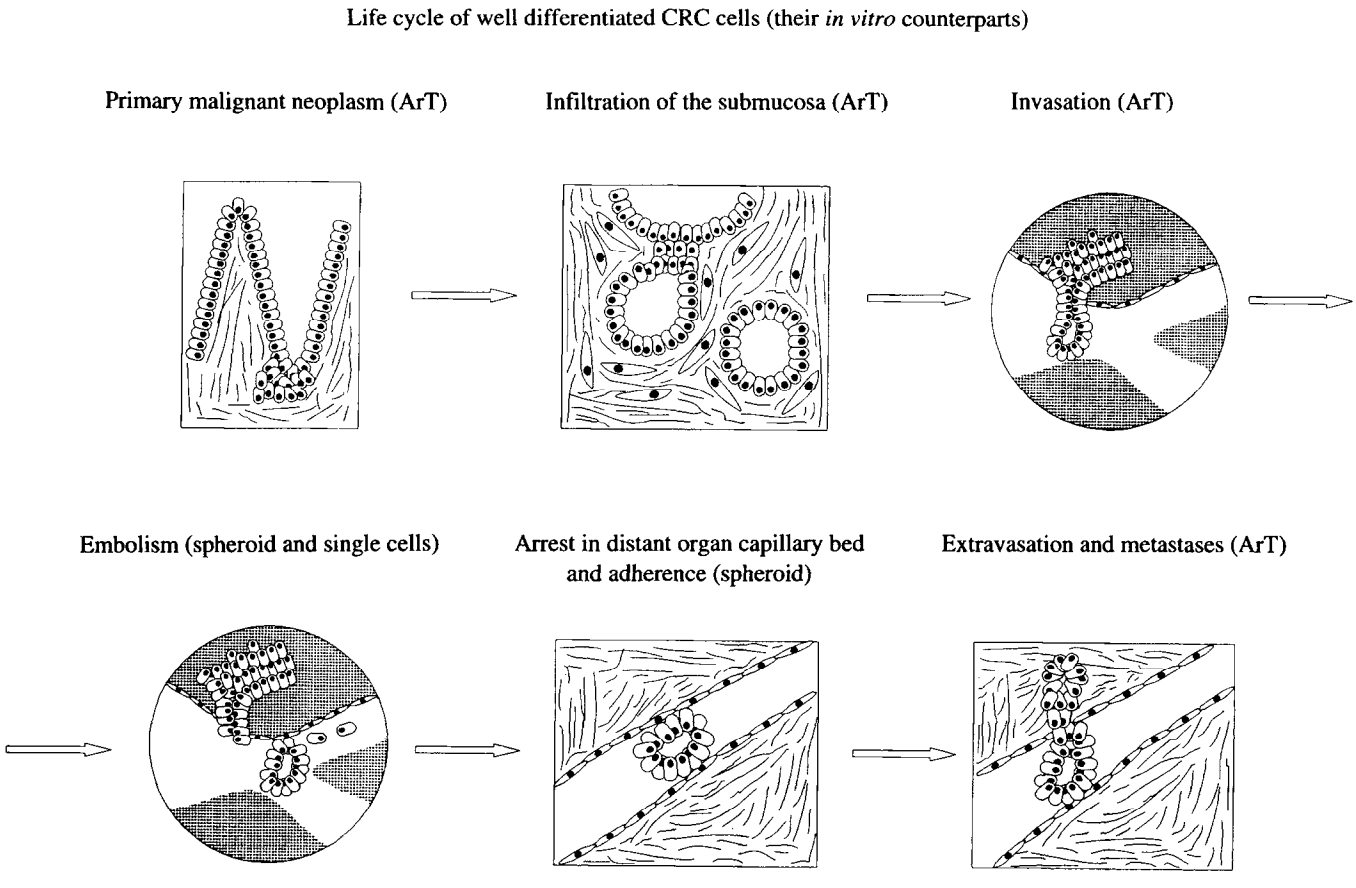

Figure 4. Diagrammatic comparison showing similarities of tumour cell arrangement *in vitro* and in different compartments of the human body. When tumour cells start to grow they first have to invade the submucosa, forming pseudoglandular structures that have contact with the tumour stroma by their basal cell membrane, identical to the situation of ArTs *in vitro*. After invading the blood or lymph vessels, they lose contact with the stroma and start to form floating cell aggregates comparable with tumour cell spheroids *in vitro*. The arrest in the capillary bed is followed by invasion of the tissue at the site of metastases. With the cells once again in contact with the stroma, the tumour cells can reorganise their structure to form pseudoglands.

distinct poles of polarised cells [12]. Therefore, the development of cytotoxic agents would probably be more effective if the details of the architecture of given tumour cells were taken into consideration.

For effector cells, the situation is completely different depending on whether they interact with tumour cell spheroids, consisting only of tumour cells, or ArTs composed like a primary tumour. LAK-cells added to spheroids immediately had close contact with the tumour cells. In contrast, LAK-cells interacting with ArTs first had to infiltrate via the stroma before they could

interact with the tumour cells. Since not all LAK-cells were able to infiltrate the ArTs with the same effectiveness, only a limited number of LAK-cells interacted with tumour cells. LAK-cells, however, can be influenced by factors secreted by fibroblasts, e.g. IL-1b, and/or from the structure of the ECM. In addition, the process of infiltration needs time in which the secreted factors from the tumour cells could influence the LAK-cells. Many authors have shown that tumour cells produce immunosuppressive factors [13–15] and are able to decrease the pH, which suppresses LAK-cell activity [16], and this may be an impotant escape mechanism in ArTs and primary tumours. However, it is unlikely that the release of suppressive factors or the depression of extracellular pH is of use when single tumour cells or tumour cell aggregates enter the circulation and travel to form distant metastases, because in the circulation the buffer function of the blood should neutralise the acid environment of the tumour cells, should it exist. Similarly, immunosuppressive factors will be diluted and may not work so quickly in order to protect tumour cells from the immune system. Therefore, the change of tumour cell architecture may be a more efficient escape strategy. A first step here would be to reduce the presence of adhesion molecules needed for a lymphocyte attack. Cepek and associates [5] showed that lymphocytes adhered to tumour cells more effectively on the basolateral cell membrane than on the apical membrane, and they suggested that this could be due to differential expression of adhesion molecules. A second step in tumour cell escape would be the expression of new molecules in high concentrations, for instance glycoproteins, which are able to protect the tumour cells from an effector cell attack. Indeed, recently we found that CEA expression of CRC cell spheroids correlates with their resistance to LAK-cell lysis [3]. This, together with the finding that CEA is mainly expressed on the apical membrane of differentiated CRC-cells [11], indicates that CEA can protect tumour cells only when they are arranged in a spheroid-like structure, i.e. unpolarised, but not when they form pseudoglands. Considering all these data, our conclusion is that tumour cells can use the change in tissue architecture as another, poorly understood escape strategy which enables them to adapt to a distinct environment they might be exposed to in different parts of the human body.

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Acknowledgements—The authors wish to thank M. Polack and F. Jehle for their excellent technical assistance. This work was supported by Deutsche Krebshilfe e.V.