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The microscopic anatomy of experimental rat CC531 colon tumour metastases: Consequences for immunotherapy?

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Abstract

The colon adenocarcinoma cell line CC531 was adopted as a model for immunotherapeutical treatment of experimental colorectal metastases in a syngeneic rat model. We studied the presence and localization of T and natural killer cells, vessels and matrix proteins in *in vivo* growing CC531 tumours by immunohistochemistry. CC531 tumours were induced either in the lungs by injecting CC531 tumour cells into a tail vein or in the liver by injection of CC531 tumour cells under the liver capsule or into a mesenteric vein. All 3 tumour types were composed of islets of tightly apposed tumour cells surrounded by abundantly present tumour-stroma which contained tumour vessels and matrix proteins. Some of these matrix proteins, especially laminin and collagen IV formed a basal membrane-like structure around the tumour nodules. This structure was most pronounced in mesenteric vein-induced liver tumours and less prominent in subcapsular-induced liver tumours and tail vein-induced lung tumours. Tumour-infiltrating lymphocytes of both T and natural killer cell origin were found in the tumours, but predominantly in the tumour stroma, separated from the islets of tumour cells by the basal membrane-like structure. We hypothesize that the matrix proteins of these tumours play an ambivalent role: they may provide a substratum for migration of effector cells into the tumour stroma but may also provide a barrier preventing direct contact between tumour target cells and immune effector cells.

Abbreviations: BM – basal membrane; BSA – bovine serum albumin; CM – complete medium; DAB – di-amino carbazole; HBSS – Hanks' Balanced Salt Solution; HRP – horse radish peroxidase; IL – interleukin; (A-)NK – (activated) natural killer; PBS – phosphate buffered saline; SCI – subcapsular-induced; MVI – mesenteric vein-induced; RT – room temperature; TVI – tail vein induced

Introduction

Treatment of metastases derived from colorectal carcinomas still forms a major therapeutic challenge in the clinic. Immunotherapy may provide new methods to combat these metastases but extensive pre-clinical analysis is necessary for an optimal translation to the patient. The chemically induced rat colon adenocarcinoma CC531 [24], which is syngeneic with the Wag rat strain has been shown to share many characteristics with human colon adenocarcinomas [32] and may therefore be a useful model to test new immunotherapeutic approaches.

We are currently investigating in our laboratory two therapeutic approaches, including the use of adoptively transferred lymphocytes (i.e. T cells or natural killer (NK) cells) and the use of interleukin 2 (IL-2). In both approaches, the anti-tumour effect is likely to be performed by either the adoptively transferred cells or by endogenous effector cells. To achieve this task, we envision that the effector cells arrive at the tumour site via the blood stream, extravasate through the endothelium, and then migrate via the extracellular matrix towards the tumour cells after which tumour cell-to-immune cell contact is established. Assuming that this series of events is indeed necessary, the success of immunotherapy, whether based on transfer of effector cells or administration of cytokines, is likely to depend on the accessibility of the malignant cells within the tumour by the effector cells. A recent paper by Mukai and co-workers demonstrated that infiltration of tumours by adoptively transferred effector cells – in this case cultured, lymph node derived T cells – may indeed be necessary for anti-tumour effects [25].

Others and we have previously shown that the number of effector cells that reaches a tumour is highly dependent

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on the location of the tumour and on the route of administration of the effector cells [1, 2, 11, 22]. The accessibility of the tumour itself is likely to depend on the extent of vascularization of the tumours and the presence, composition and localization of matrix proteins in the tumour stroma [27]. Furthermore, the number and localization of endogenous, tumour-infiltrating T-and NK cells will probably be of importance for the therapeutic application of cytokines.

In the present study we have investigated the presence and localization of matrix proteins, blood vessels and T and NK cells in CC531 tumours. Liver tumours, either subcapsularly-induced or induced by injection via a mesenteric vein, and lung tumours, induced by injection of tumour cells via a lateral tail vein, were subjected to these studies. Immunohistochemical parameters were compared in these 3 tumour types to see if tumours from the same cell line, but differently induced, have different characteristics which could have implications for immunotherapy.

Materials and methods

Animals

Male Wag-Rij rats, a Wistar-derived inbred strain, were purchased from either Charles River (Schulzfeld, Germany) or from Harlan (Zeist, The Netherlands) and used at 4–5 months of age, weighing approximately 260 grams. The animals had free access to standard food pellets and water. All animal experiments were approved by the local committee for animal welfare.

Induction of liver-and lung tumours

The CC531 cell line, derived from a dimethyl hydrazine-induced Wag rat adenocarcinoma of the colon [24] was cultured in complete medium (CM) which consisted of RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine, 50 μg/ml streptomycin and 50 U/ml penicillin (all from Gibco, Paisley, UK). Cells were maintained by serial passage. Tumour cells were harvested with a solution of 0.1% (w/v) EDTA and 0.25% (w/v) trypsin in Hank's Balanced Salt Solution (HBSS, supplied by Sigma, St. Louis, Missouri, USA), washed three times in 0.9% (w/v) NaCl solution buffered with 1.4 mM phosphate (PBS) and adjusted to a suspension containing 1.0×10^6 viable tumour cells (trypan blue exclusion test) per ml. All surgical procedures were performed under clean but not sterile conditions. The rats were anesthetized using halothane. For the induction of liver tumours, the liver was exposed by laparotomy using a median incision. For induction of subcapsular-induced (SCI) liver tumours, 50 μl of tumour cell suspension containing 50×10^3 tumour cells was injected subcapsularly (4 tumours per liver) at 2 places in both the left and right main lobes of the liver. Mesenteric vein-induced (MVI) liver tumours were created by the injection of 1×10^6CC531 cells via a mesenteric vein. Subcapsular tumours were allowed to grow for 5 days and mesenteric tumours for 10 days. For induction of CC531 lung tumours, rats were first injected with 30 mg cyclophosphamide in 1 ml PBS into the peritoneal cavity and 1 day thereafter with 2.5×10^6 CC531 cells in 1 ml RPMI 1640 in a lateral tail vein. Lung tumours were allowed to grow for 12 days.

Antibodies

The primary antibodies used are summarized in the following table:

Antibody and isotype	Recognizes	Species	Supplier
CC52 IgG1	CC531 cells	mouse	1)
3.2.3 IgG1	rat CD161A	mouse	2)
R73 IgG1	rat TCR$\alpha\beta$	mouse	3)
RECA 1 IgG1	rat endothelium	mouse	4)
polyclonal	collagen type I	goat	5)
polyclonal	collagen type III	goat	5)
polyclonal	collagen type IV	rabbit	6)
polyclonal	collagen type VI	rabbit	7)
polyclonal	laminin	rabbit	8)
polyclonal	fibronectin	goat	9)

1) Own department [4, 32].
2) Own department, gift from Dr W.H. Chambers, Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania, USA [5].
3) Own department, gift from Dr Th. Hünig, University of Würzburg, Germany [17].
4) Gift from Dr. A.M. Duijvestijn, Department of Immunology, University of Limburg, Maastricht, The Netherlands [8].
5) Produced by Sera-Lab, supplied by Sanbio, Uden, The Netherlands.
6) Produced by HEYL, Berlin, Germany, and supplied by Sanbio, Uden, The Netherlands.
7) Produced by Telios, San Diego, USA, and supplied by Sanbio, Uden, The Netherlands.
8) Produced by HEYL, Berlin, Germany, and supplied by Sanbio, Uden, The Netherlands.
9) Produced by Sigma, St. Louis, Missouri, USA, and supplied by Brunschwig, Amsterdam, The Netherlands.

Immunohistochemistry

Five-μm-thick cryostat (Reichert Jung 2800 Frigocut, Leica, Nussloch, Germany) sections were cut from the snap frozen tumour tissue. Sections were air-dried for at least 16 h at room temperature (RT). Next, they were fixed in acetone for 10 min and washed twice in PBS. All dilutions of antibodies and conjugates were performed with PBS containing 1% (w/v) bovine serum albumin (BSA, Boehringer Mannheim, Germany). The tissue sections were incubated for 60 min with a previously determined optimal concentration of protein A purified primary antibody. After incubation with the primary antibody, the sections were washed in PBS three times for 5 min. In case of mouse primary antibodies, the sections were incubated for 30 min with horse radish peroxidase (HRP)-conjugated rabbit anti-mouse Ig (dilution 1:100) and subsequently with HRP-conjugated swine anti-rabbit Ig (dilution 1:50, both obtained from DAKO, Glostrup, Denmark) and subsequent washes in PBS. In case of rabbit or goat polyclonal antibodies as primary antibodies, the sections were incubated with HRP-conjugated swine anti-rabbit Ig or HRP-conjugated rabbit anti-goat Ig (dilution 1:100,

obtained from DAKO). Visualization of immune complexes was performed by a 10 min incubation with a di-amino carbazole (DAB) substrate in a buffered TRIS-HCl (pH = 7.6) solution containing 1.8×10^{-3} % (v/v) hydrogen peroxide. For immunohistochemical single-color stainings, the sections were counter stained using hematoxylin, dehydrated and mounted using Pertex (Histolab, Göteborg, Sweden).

In case of double stainings, the sections were washed twice in PBS and subsequently incubated for 30 min with a second primary antibody. After 3 wash steps with PBS, the sections were incubated for 30 min with either HRP-conjugated rabbit anti-mouse Ig, with HRP-conjugated swine anti-rabbit Ig or with HRP-conjugated rabbit anti-goat Ig (all DAKO). The immune complexes were visualized by a 12 min incubation step in a buffered TRIS-HCl (pH = 7.6) solution containing per 100 ml: (1) 40 mg 4-chloro-1-naphtol (Merck, Darmstadt, Germany) dissolved in 200 μl di-methyl formamide (Baker B.V., Deventer, The Netherlands) and 300 μl ethanol (Merck); and (2) 200 μl of a 30% (v/v) H_2O_2 solution (Merck). The sections were slightly counter stained using nuclear fast red and mounted using Kaiser's Glycerine (Merck). Three control sections per tumour were included in which either one or both primary antibodies were omitted.

Results

Liver tumours and lung tumours: macroscopic and microscopic anatomy

At macroscopic level, tail vein-induced (TVI) lung tumours appeared as white nodules in the lung parenchyma. On cross sections the tumour areas varied from approximately 0.04 mm^2 to 0.12 mm^2. The subcapsularly-induced (SCI) liver tumours appeared as white nodules on the liver surface with some scar tissue due to the needle which was used for injection of tumour cell suspension. On cross sections, the tumour areas were approximately 1.9 mm^2. Mesenteric vein-induced (MVI) liver tumours were randomly distributed throughout the liver parenchyma. Their sizes on cross sections varied considerably from 0.03 mm^2 to 1.8 mm^2 to large confluent areas of tumour tissue.

TVI lung tumours (Figure 1a), SCI liver tumours (Figure 1b), and MVI liver tumours (Figure 1c) showed the same microscopic aspect: the tumours were composed of islets of closely apposed tumour cells which were surrounded by a stromal component. Both types of liver tumours revealed an abundant stromal compartment whereas the TVI lung tumours had a sparse stromal compartment. In all 3 tumour types the islets of tumour cells showed a large variation in size. In MVI liver tumours the tumour cell islets had a glandular-like shape whereas the tumour cell islets of SCI liver tumours and TVI lung tumours had a less regular shape.

Matrix composition

Analysis by immunohistochemistry revealed the presence of collagen type I, III (Figures 2a, c, e), IV (Figures 2b, d, f), VI, laminin and fibronectin in all 3 tumour types, but in the liver tumours (Figure 2c–f) a larger amount of matrix proteins was present than in TVI lung tumours (Figures 2a, b). Matrix proteins were present in the stromal compartment between the tumour nodules but not in the tumour nodules themselves. The tumour nodules were tightly surrounded by a basal membrane (BM)-like structure consisting of laminin and collagen IV. This was most pronounced in the MVI liver

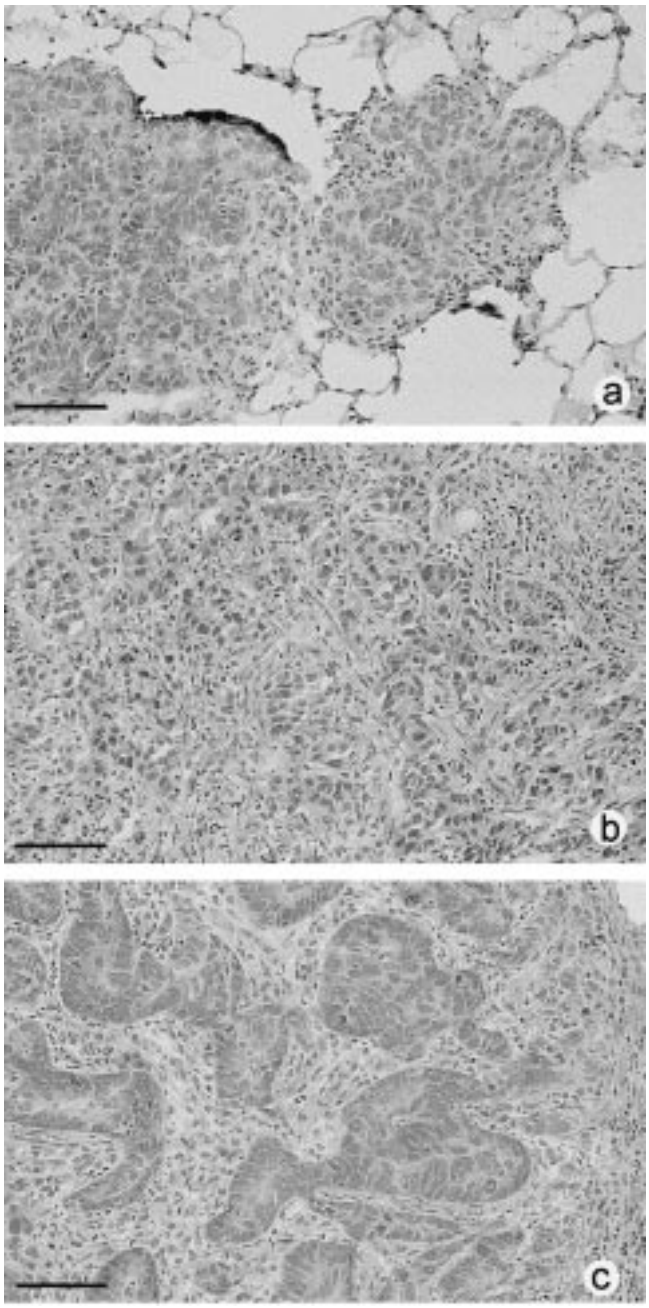

Figure 1 Typical aspect of haematoxylin and eosin-stained sections of tail vein-induced (TVI) lung tumours (a), subcapsularly-induced (SCI) liver tumours (b), and mesenteric vein-induced (MVI) liver tumours (c). All tumour types were composed of islets of closely apposed tumour cells surrounded by a stromal component. Both types of liver tumours revealed an abundant stromal compartment whereas the TVI lung tumours had a sparse stromal compartment. The islets of tumour cells, which showed a large variation in size in all 3 tumour types, had a glandular-like shape in MVI liver tumours (c) whereas the tumour cell islets of TVI lung tumours (a) and SCI liver tumours (b) had a less regular shape. Bar indicates 150 μm.

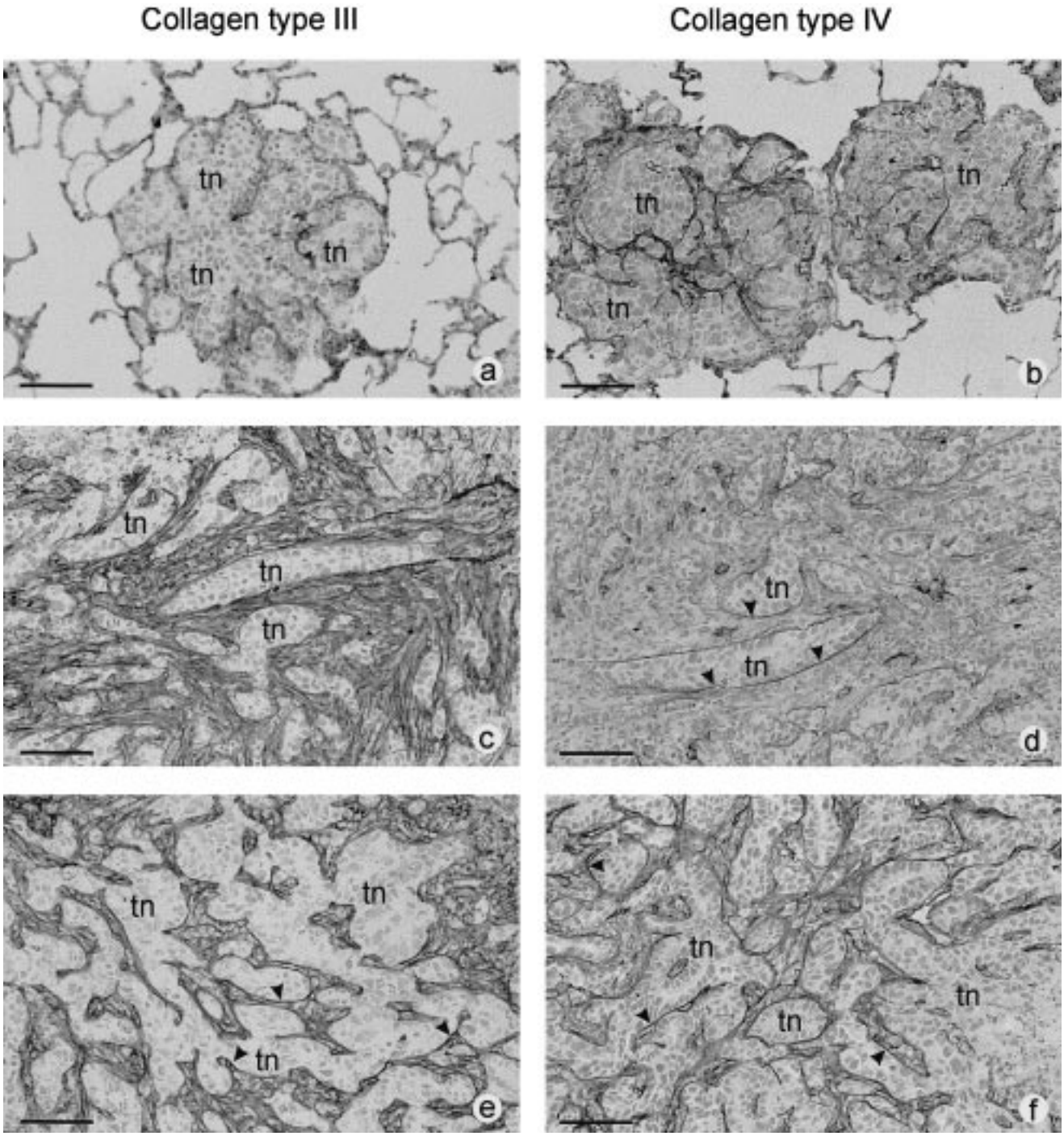

Figure 2 Tail vein-induced (TVI) lung tumours (a, b), subcapsular-induced (SCI) liver tumours (c, d) and MVI liver tumours (e, f) were stained for collagen type III (a, c, e) and collagen type IV (b, d, f) using immunohistochemistry. The tumour nodules (indicated by tn) were tightly surrounded by a BM-like structure (arrowheads) consisting of laminin (not shown) and collagen IV (b, d, f). In MVI liver tumours the BM-like structure did also contain other matrix components like collagen type III (e, indicated by arrowheads). Bar indicates 150 μm.

tumours and less in the SCI liver tumours and TVI lung tumours. In SCI liver tumours the surrounding proteins were less continuous than in MVI tumours and in the TVI lung tumours the matrix proteins surrounding the tumour nodules in general had a thinner appearance. In MVI liver tumours the BM-like structure did also contain other matrix components like collagen type III (Figure 2e).

Vascularization of CC531 tumours

We studied the presence of vessels in the 3 different tumour types. Tumour-associated vessels were stained using RECA 1 [8], a mouse monoclonal antibody which has been shown to specifically recognize rat endothelium.

In all 3 tumour types vessels were found, as revealed by immunohistochemistry (Figures 3a, b, c). The endothelial cells were confined to the tumour stroma and were not present in the tumour nodules which suggested that direct

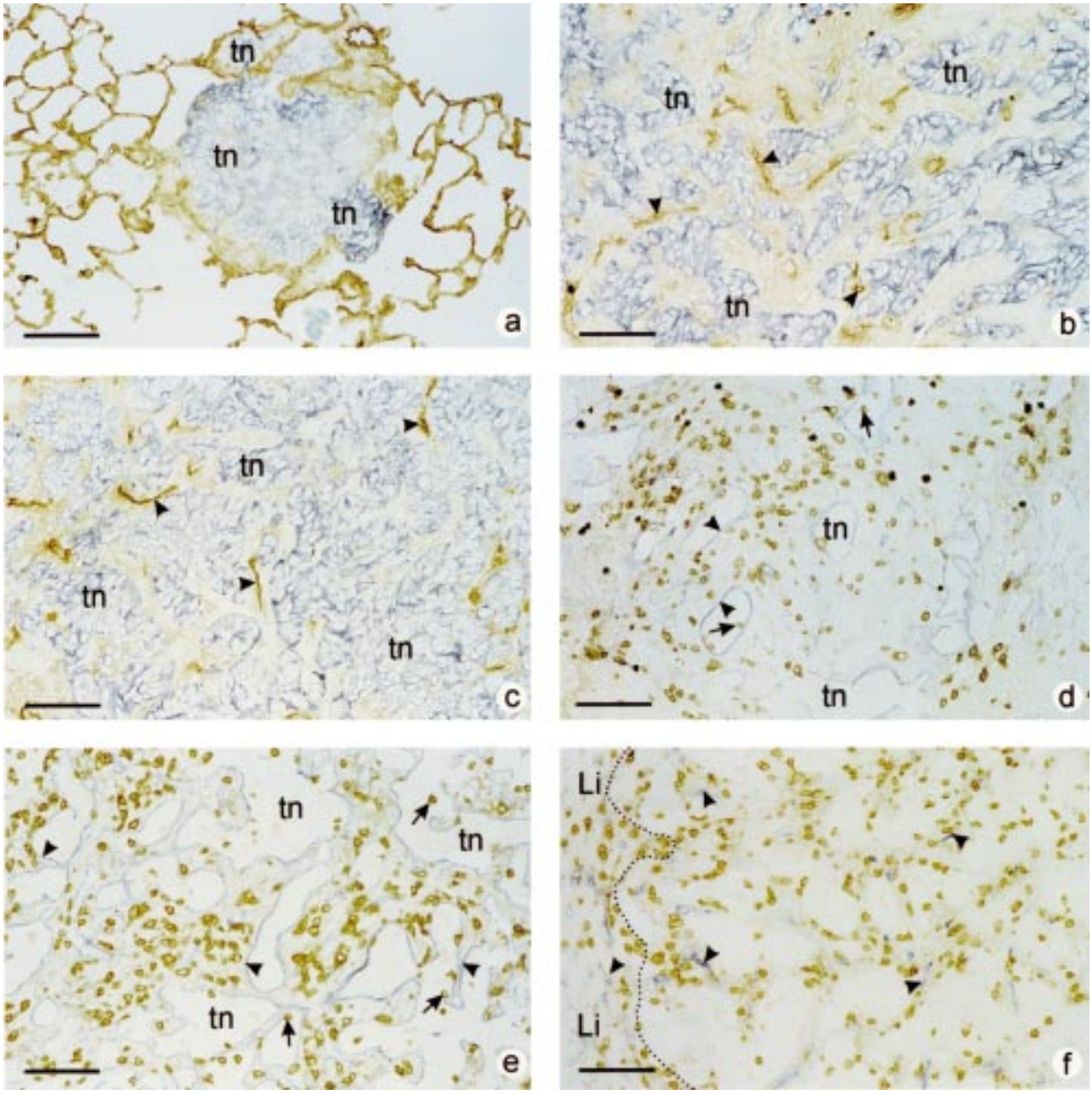

Figure 3 Sections of tail vein-induced (TVI) lung tumours (a), subcapsular-induced (SCI) (b)-and mesenteric vein-induced (MVI) (c) liver tumours were double-stained for vessels (brown, indicated by arrowheads) and tumour cells (blue) using immunohistochemistry. Vessels were confined to the tumour stroma and not present in the tumour nodules. Sections of SCI liver tumours (d) and MVI liver tumours (e) were double-stained for T cells (brown) and collagen type lV (blue). T cells were predominantly located in the tumour stroma and not in direct contact with tumour cells in the tumour nodules (tn). These effector cells were separated from the tumour nodules by a BM-like condensation of matrix proteins surrounding the tumour nodules (indicated by arrowheads). Occasionally a T cell was found in the tumour nodules (indicated by arrows). A section of a MVI liver tumour, double-stained for T cells (brown) and vessels (blue-stained structures, indicated by arrowheads) is shown in (f). A part of the liver parenchyma (indicated by Li) surrounding the tumour is visible. The border between the tumour tissue and the liver parenchyma is indicated by a dotted line. Bar indicates 150 μm.

access to the tumour cells is only provided via the tumour stroma. In lung tumours, the majority of vessels was found in the periphery and not in the center of the tumour (Figure 3a) but variation was observed in this pattern as some tumours had also vessels in central parts. In SCI (Figure 3b) and MVI liver tumours (Figure 3c), the vessels were observed both in the periphery but also in central parts of the tumour.

Infiltration of the tumours by T and NK cells

Tumours were stained for T and NK cells in order to precisely locate these effector cells in the different tumour types. In all 3 tumour types, both NK cells and T cells were observed in an irregular distribution pattern. T cells, and, to a lesser extent, NK cells clustered in large numbers at the periphery of the tumours. The number of T cells was higher than the number of NK cells. As can be seen in Figures 3d

and 3e, the majority of the T cells was located in the tumour stroma and not in direct contact with tumour cells. NK cells showed an identical distribution pattern (not shown). Double stainings for T or NK cells and matrix proteins showed that these effector cells were separated from the tumour nodules by the BM-like condensation of matrix proteins surrounding the tumour nodules. Occasionally such an effector cell was found in the tumour nodules (Figure 3d, e).

Double stainings for T or NK cells and endothelium revealed that the majority of the lymphocytes was present in the tumour stroma without any adjacent vessel (Figure 3f). This demonstrated that most of the T and NK cells had extravasated and had migrated into the tumour stroma.

Discussion

The feasibility of treatment of colorectal cancer metastases using cytokines like IL-2 and/or the adoptive transfer of IL-2 cultured NK (A-NK) cells [11, 12] or T cells [3] will probably depend on the ability of these effector cells to reach the tumours and to establish contact with the malignant cells. In a recent paper, Mukai et al. demonstrated in a syngeneic mouse lung metastasis model that the presence of adoptively transferred T cells inside the tumour nodules is indeed a prerequisite to attain significant anti-tumour effects [25]. If the local presence of effector cells is indeed necessary, then factors such as tumour vascularization and tumour content and composition of matrix proteins may be important factors to evaluate. Consequently, we have analyzed these factors in the CC531 model of colon cancer.

In the CC531 model, both SCI and MVI liver tumours and TVI lung tumours appeared to be composed of islets of clustered tumour cells which were surrounded by matrix. In all 3 tumour types, the matrix was shown to contain collagen types I, III, IV, VI, fibronectin and laminin. In the CC531 TVI lung tumours, less matrix was observed than in liver tumours but it must be emphasized that the TVI lung tumours were much smaller than both types of liver tumours. It is therefore possible that the TVI lung tumours will reach a more organized, glandular-like architecture with more matrix formation after prolonged outgrowth.

Elimination of tumour cells by endogenous or adoptively transferred effector cells is probably dependent on direct contact between the target- and effector cells. In this context, it is remarkable that the majority of infiltrating immune cells – as we also showed in a previous paper [12] – was localized in the tumour stroma and not in contact with the tumour cells. This discrepancy might be explained in several ways. It is possible that there is an insufficient chemotactic stimulus attracting the effector cells towards the tumour cells. Alternatively, it might be that the effector cells that reached the tumour nodules died fast or that the tumour cells could not be reached by the effector cells due to fibers of matrix which tightly surrounded the tumour nodules. This BM-like structure around the islets of tumour cells, which was previously described by Griffini et al. [10], may have limited the accessibility of the tumour cells. On the other hand, a recent paper by Goldfarb et al. demonstrated – *in vitro* but also *in vivo* in our rat model – that the BM-like barrier might be degraded by matrix metalloproteinases which were secreted by *in vitro* cultured, IL-2 activated NK cells [19]. This may suggest either that the BM-like structure was not a real obstacle for the effector cells or that the amount and/or quality of BM-degrading enzymes secreted by most of the effector cells were insufficient.

The BM-like structure in CC531 tumours was less pronounced in the SCI liver tumours and in the TVI lung tumours than in MVI liver tumours. This implies that the location (lung versus liver) and the method of induction (MVI versus SCI) may influence the amount and distribution of matrix proteins in the tumours. A similar distribution pattern of immune cells [6, 7, 26] and matrix proteins [9, 13, 23] like we found in our CC531 tumour model has also been described for primary human colon tumours [9, 13, 23], for lymph node metastases [23] and for other types of human primary malignancies [14, 16, 20, 30, 34]. If matrix proteins influence accessibility of tumours for lymphocytes, one would expect that susceptibility for immunotherapy of these tumours depends – at least in part – on the anatomy of the matrix meshwork. In that case, the differences in matrix composition like we found in the 3 different models might be relevant for humans. In this context, it is also interesting to note that in previous experiments, a much higher amount of adoptively transferred A-NK cells reached CC531 lung tumours [21] than liver tumours [11]. This might be explained by the described differences in matrix composition of these tumour types. Alternatively, less A-NK cells may reach liver tumours as compared to lung tumours as the majority of A-NK cells in the CC531 liver tumour experiment seemed to be trapped in the portal tracts which might have prevented further migration towards the tumours.

In contrast to our approach, most attention in the literature with respect to tumour cells and matrix proteins has been directed towards the relation between the presence of matrix proteins in tumours and tumour cell differentiation, tumour stage, metastasis, and prognosis. The investigations consistently report a positive correlation between deposition of BM-like structures and an increasing degree of differentiation of the tumour cells in humans [9, 13, 23]. A similar phenomenon has been described in a rat model [29]. Controversy exists with respect to the predictive value of BM-like deposits for the development of metastases, tumour stage and survival in humans. For instance, Hida et al. were not able to demonstrate a correlation between the presence of this structure in the primary tumour and the stage of the tumour [15]. Furthermore, their results suggested that BM-preservation may promote the development of liver metastases [15]. Other papers, however, demonstrated an inverse correlation between the presence of a BM-like structure and the presence of metastases [9] or survival [9, 13, 28]. The precise relationship between presence of BM-like matrix deposits and prognosis is apparently not clear. If immune cells are important to eliminate developing tumours, one would expect that the absence of a BM-like structure surrounding the tumour cells would favor the immunologic control.

Another important item when considering adoptive immunotherapy is the number and localization of vessels in the tumour, which is probably a prerequisite for initial arrival of effector cells in the tumour. Experiments by Nannmark and co-workers demonstrated in a syngeneic mouse tumour model that infiltration of lung metastases by adoptively transferred, A-NK cells was correlated with the extent of vascularization of these tumours [27]. This demonstrated that the presence of vessels in the tumour may determine the success of adoptive immunotherapy. We demonstrated in all types of CC531 tumours the presence of vessels but solely in the stromal part. Thus, the effector cells will not get in contact with the malignant cells immediately following their trans-vascular diapedesis. Rather, they must migrate across the stroma, possibly using the stromal matrix fibers as a substratum for migration. Both *in vitro* and *in vivo* studies have demonstrated that this may indeed be the case [12, 18, 31, 33].

The studies on distribution and composition of matrix proteins and on vascularization which are described in this paper are confined to a single time point of tumour growth. It might be that during tumour progress, conditions with regard to matrix distribution and neovascularization show changes. This may have consequences for the accessibility of the tumour cells for the effector cells and thus probably for anti-tumour effects. Furthermore, since CC531 liver tumours grow relatively rapidly compared to human tumours, it might also be that the growth rate limits the amount of effector cells and neoangiogenic vasculature that penetrates the tumour. Therefore, it would be of interest to perform similar studies in a model with slowly growing adenocarcinomas of colorectal origin.

In summary, we have demonstrated that 3 different types of *in vivo* growing CC531 tumours – i.e. SCI and MVI liver tumours and TVI lung tumours – were composed of islets of tightly apposed tumour cells surrounded by matrix proteins. We hypothesize that these matrix proteins play an ambivalent role: they may provide a substrate for migration in the tumour stroma but around the tumour nodules a barrier to establish contact with the tumour cells. Therefore, more attention should be focused on the relation between tumour infiltrating effector cells and the local expression of matrix proteins. The presence of a tight BM-like structure of matrix proteins may perhaps in part explain the failure of previous clinical immunotherapeutical trials. In that case, the ultimate challenge in immunotherapy may not only be the development of cells capable of eliminating tumour cells but also to design strategies which may overcome this matrix barrier.

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