

Monoclonal antibodies for immunodetection of fibrin deposits on cancer cells

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Summary. The progression of a tumor from benign to malign and localized to invasive and metastatic growth is the major cause of poor outcome of therapy in cancer patients. The deposition of fibrin along with other pro-coagulant molecules into the extracellular matrix obviously serves as a scaffold to support proliferation, migration and tumor cell growth as well as protection against the immune system.

The use of antibodies as agents for the immunodetection of fibrin deposits *in vivo* has been hampered by anti-fibrin cross-reactivities with fibrinogen. For the immunohistochemical detection of fibrin we used highly specific monoclonal antibodies to a synthetic fibrinunique peptide, because the fibrin molecule shares many epitopes with fibrinogen. The monoclonal antibody was applied to adenocarcinoma of the colon, mamma, pancreas, sarcoma and acute myeloic leukemia. In all tissue sections and cytospin preparations fibrin was identified in a direct apposition to the surface membranes of carcinoma and sarcoma cells, predominantly at the host-tumor interface and also in regions directly adjacent to zones of angiogenesis, whereas normal cells and tissue showed no deposits of fibrin. The findings will be supported by investigations that factors and components of the coagulation system could be detected in the tumor stroma and tumor cells. These factors are obviously produced and secreted by the malignant cells and deposited together with fibrinogen into the extracellular matrix. Our results show that basically all malignant cells examined, independently of ectodermal or mesenchymal derivation, themselves are the origin of hypercoagulability and fibrinolytic system inhibition.

Key words: Fibrin, Immunocytochemistry, Cancer cells, Hypercoagulability, Metastasis

Introduction

It is well known that cancer increases the risk of venous thromboembolism (Falanga, 2004). The first description of venous thrombosis as a complication of malignant tumors by Trousseau dates back to 1865. Later, in 1878, Billroth describes cancer cells of different tumors within thrombi and interprets these findings as evidence of the spread of tumor cells by thrombemboli. In the last years several studies had been published about venous thromboembolism and cancer (Jang et al., 2006). Patients with mucin-secreting adenocarcinomas of the gastrointestinal tract, ovary or lungs are believed to represent a particularly high-risk group for a venous thrombophlebitis.

The pathogenesis of the haemostatic disorders in cancer is complex and reflects the interaction of different mechanisms, i. e. activation of the coagulation and fibrinolytic systems, endothelial factors and activation of cellular mechanisms for promotion of clotting on the surface of monocytes and platelets. The progression of a tumor from benign to malign and localized to invasive and metastatic growth is the major cause of poor outcome of therapy in cancer patients. Very similar to a healing wound or chronic local infection, the deposition of fibrin along with other pro-coagulant molecules into the extracellular matrix serves as a scaffold to support proliferation, migration and tumor cell growth as well as protection against the immune system (Hogg, 1983; Bobek et al., 2005). The use of antibodies as agents for the immunodetection of fibrin deposits *in vivo* has been hampered by antifibrin cross-reactivity with fibrinogen.

We used monoclonal antibodies which bind specifically to fibrin even in the presence of human fibrinogen (Hui et al., 1983). Therefore it was the goal of this study to investigate the immunohistochemical localization of fibrin with different kinds of tumors of endothelial origin (adenocarcinomas), the haematological system and sarcoma (Nakstad and Lyberg, 1991).

Materials and methods

For the immunohistochemical demonstration of fibrin we used special monoclonal antibodies to a synthetic fibrinunique peptide because the fibrin molecule shares many epitopes with fibrinogen. These antibodies which recognized the synthetic fibrin epitope bind to fibrin exclusive of fibrinogen. The heptapeptide of the amino terminus of fibrin's β -chain was synthesized to serve as a fibrinunique antigen, since we reasoned that the amino terminus is exposed after thrombin cleavage. Along with the first seven amino acids of the β -chain cysteine was placed at the carboxyl terminus to permit unidirectional attachment of the synthetic peptide to maleimidobenzoylated keyhole limpet hemocyanin (MB-KLH). It was assumed that after the β -peptide had reacted with MB-KLH all peptide amino termini would be uniformly oriented away from the protein carrier in a manner most analogous to that of fibrin β -chains (Hui et al., 1983; Kaufmann et al., 1994).

Immunohistochemical reactions were performed using a standardized sequence based on the streptavidin-biotin technique for detection of the appropriate biotinylated link antibody with a commercial monoclonal antibody (Immunotech Antibody E 8). All immunohistochemical procedures (see Table 1) were controlled by replacing the antibody with mouse serum (1:20). None of the controls revealed staining.

The described monoclonal antibody was applied on 36 histopathological confirmed tumors of different

tissues, such as adenocarcinoma of colon, mamma, ovary, prostate, pancreas and stomach. Additionally, to represent the basic kinds of tumors we used this antibody at sarcoma and acute myeloid leukemia.

Before the biopsies and extirpations of the tumors all patients were diagnosed by magnetic resonance imaging (MRI). This study, in which human tissues were collected from different neoplasia used for routine histopathological diagnosis, has been approved by the local ethic committee (Institut für Pharmakologie und Toxikologie, Universität Würzburg) and all patients gave written informed consent.

Results

All tumor cells of different malignoma *in vivo* and tumor cell lines obviously express fibrin as shown by immunohistochemistry. The slides showed clearly that all malignant cells, independently of ectodermal or mesenchymal derivation are the origin of hypercoagulability and fibrinolytic system inhibition. In Figures 1 and 2 the tissues are presented with the isotype control slides and the corresponding immunohistochemical staining.

Discussion

In previous investigations on histological sections we observed clots of pure fibrin around and in tumor tissue. But in all these sections it was not possible to detect a clear function of this fibrin.

Therefore we applied monoclonal antibodies for immunodetection of fibrin deposits on different malignant cells.

In all tissue sections fibrin was identified in a direct apposition to the surface membranes of carcinoma and sarcoma cells, predominantly at the host-tumor interface and also in regions directly adjacent to zones of angiogenesis, whereas normal cells and tissue showed no

Table 1. Immunohistochemical sequence with human cold agglutinins as primary antibodies.

Steps	Reagents	Conditions	Dilution/Diluent
Blocking of peroxides	3% Hydrogen peroxide in methanol	10 min. RT	Freshly prepared
Washing	0.1 M TRIS-HCL (pH 7.6)	2x5 min. RT	
Blocking endogenous IgM	Dako (A425, swine antibody)	30 min. RT	1:150/0.05 M TRIS-HCL (pH 7.6), 15 mM sodium azide, 6% BSA
Washing	0.1 M TRIS-HCL (pH 7.6)	2x5 min. RT	
Blocking reagent	Swine serum (1:20)	20 min. RT	1:20/0.05 M TRIS-HCL (pH 7.6), 15mM sodium azide, 6% BSA
Primary antibody	Immunotech E8 Anti-fibrin	24 h 4°C	Dilution see Table 1/0.05 M TRIS-HCL (pH 7.6), 15 mM sodium azide, 1.5% BSA
Washing	0.1 M TRIS-HCL (pH 7.6)	2x5 min. RT	
Link antibody	Dako (E483, swine antibody)	30 min. RT	1.25/0.05 M TRIS-HCL (pH 7.6), 15 mM sodium azide, 6% BSA
Washing	0.1 M TRIS-HCL (pH 7.6)	2x5 min. RT	
Streptavidin-HRP	Dako (P397)	30 min. RT	1:400/0.05 M TRIS-HCL (pH 7.6)
Washing	0.1 M TRIS-HCL (pH 7.6)	2x5 min. RT	
Detection	DAB-chromogen	10 min. RT	

RT: room temperature.

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deposits of fibrin (Figs. 1, 2). These findings are in accordance with former investigations performed by fluorescence labelled antibodies or polyclonal antibodies (Wojtukiewicz et al., 2001; Im et al., 2004). These results will be supported by investigations that factors and components of the coagulation system could be identified in the tumor stroma and tumor cells. Moreover, these monoclonal antibodies are valuable tools for detection, investigation and prognosis of leukemic cells (Linch et al., 1984). A lot of studies demonstrated by immunohistochemical procedures that tumor cells are producing several tissue factors and components for coagulation i.e.: factors VII, VIIIc, IX,

X, XII, XIIIa and prothrombin (Wojtukiewicz et al., 2000; Gieseler et al., 2007).

These factors are obviously produced in the malignant cells and secrete and deposit together with fibrinogen into the extracellular matrix (ECM) (Rybarczyk and Simpson-Haidaris, 2000). Specially fibrinogen was present in abundance throughout the connective tissue in breast cancer but not in non-malignant tissues (Costantini et al., 1991).

On the other hand the fibrinolytic system is obviously inhibited in the tumor tissue as well as in carcinomas or sarcomas and moderately activated in the tumoral draining vein samples detected with factors such

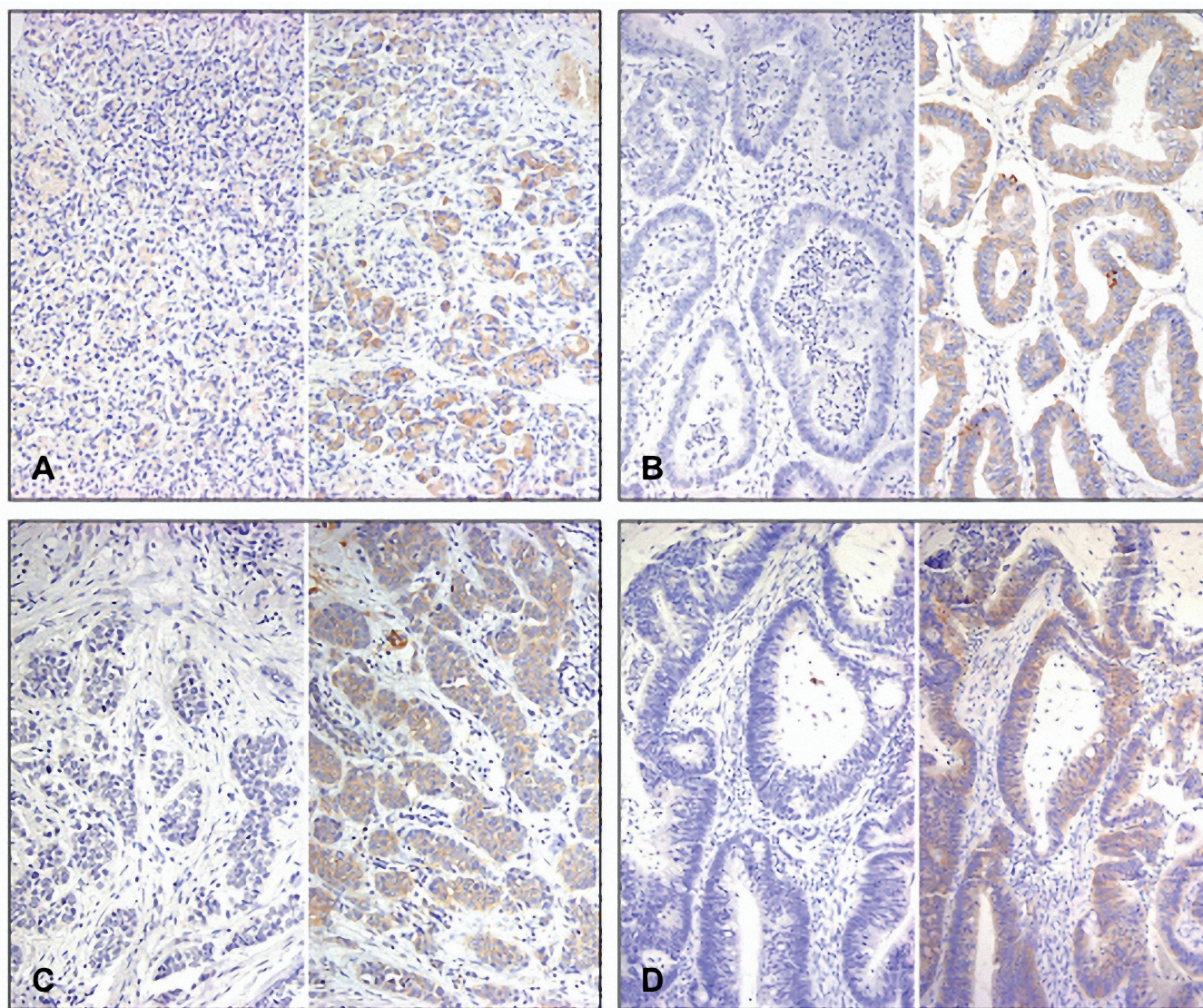


Fig. 1. Fibrin was clearly expressed on tumor cells of different carcinomas. **A.** Pancreatic carcinoma (isotype control, immunohistochemistry). **B.** Ovarial carcinoma (isotype control, immunohistochemistry). **C.** Breast carcinoma (isotype control, immunohistochemistry). **D.** Colon carcinoma (isotype control, immunohistochemistry). *In vivo* as shown by immunohistochemistry. Slides were counterstained by hematoxylin and eosin. x 20

as t-PA, D-dimer, AT III and PAI-1 (Nakstad and Lyberg, 1991; Heiss et al., 1995; Garcia-Avello et al., 2001; Palumbo et al., 2000, 2003; Palumbo and Degen, 2001).

These findings show that basically all malignant cells, independently of ectodermal or mesenchymal derivation, themselves are the origin of hypercoagulability and fibrinolytic system inhibition (high levels of thrombin-antithrombin III complexes and PAI-1), whereas in the vein a moderate activation of the fibrinolytic system (t-PA and D-dimer elevation) (Garcia-Avello et al., 2001) exists.

This complex system of hypercoagulations with all its factors and components in the tumor system and

extracellular matrix provides a scaffold against the immune system of the body. However, on the other hand the fibrinolytic system of the draining veins of the tumor, obviously promotes the releasing of the tumor cells and supports with it tumor cell spreading (Rybarczyk and Simpson-Haidaris, 2000; Garcia-Avello et al., 2001; Palumbo and Degen, 2001; Wojtukiewicz et al., 2001; Im et al., 2004; Jang et al., 2006). A systemic or local increase of the coagulation factors causes in consequence the docking and nidation of the spreading tumor cells. With the division and multiplication of these malignant cells a new metastasis grows up under the scaffold and shelter of fibrin. In accordance to these

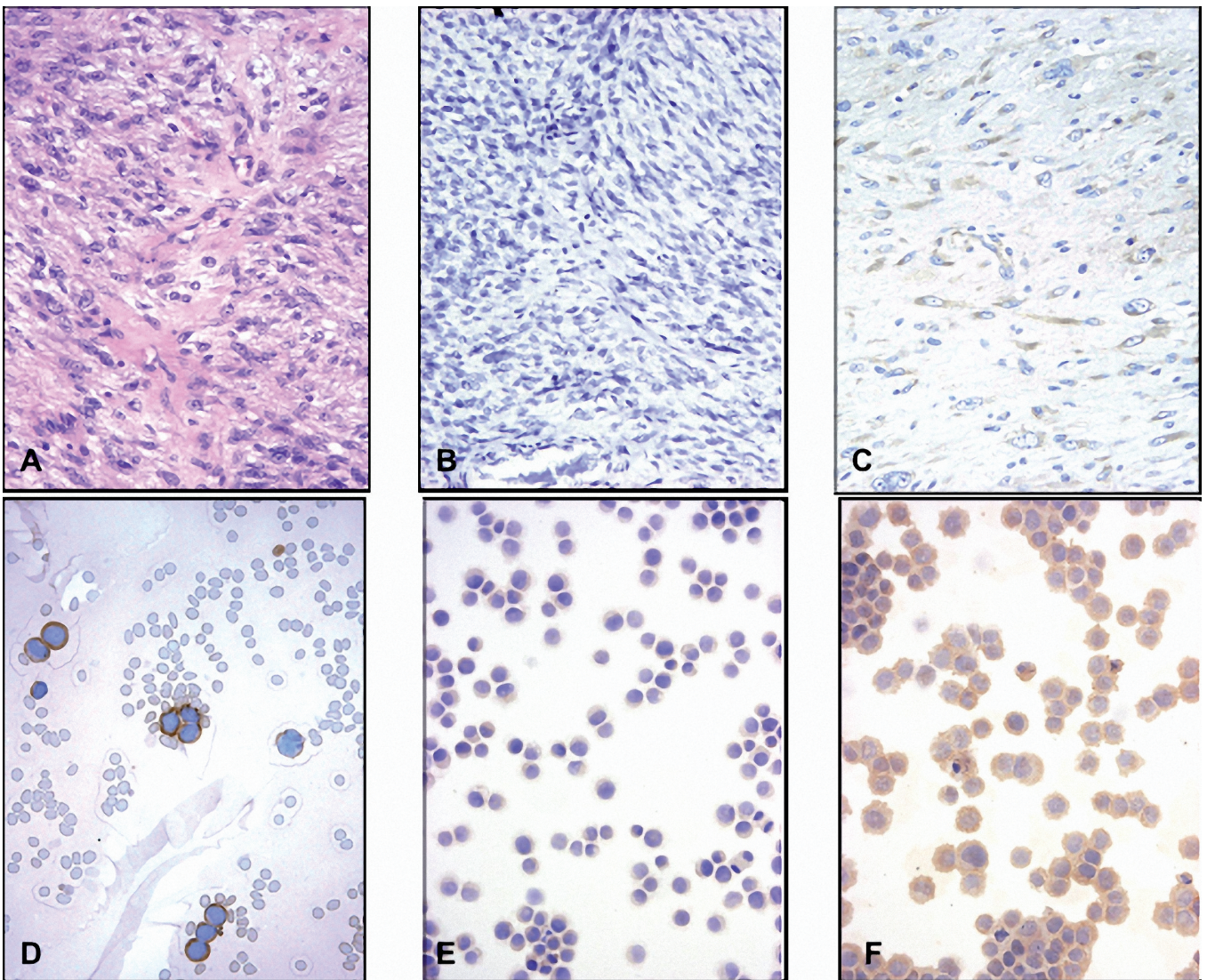


Fig. 2. Fibrin was also expressed on tumor cells of. **A** Leiomyosarcoma (HE). **B.** Leiomyosarcoma (isotype control). **C.** Leiomyosarcoma (immunohistochemistry). **D.** Acute myeloblastic cells HMO2 (immunohistochemistry). **E.** Gastric carcinoma cell line (isotype control, zytospin MKN 45). **F.** Gastric carcinoma cell line (immunohistochemistry, zytospin MKN 45). A-C, x 20; D-F, x 40

findings the intrauterine perivillous deposition of fibrin seems also to protect mother and foetus as a barrier against immunoreactions of one to another (Frank et al., 1994; Kaufmann et al., 1994; Lang et al., 1994; Svensson et al., 2004). To protect itself with an extracellular matrix and fibrin against the immune system of the body seems to be a basic condition for growth and metastasis of a malignant cell. Because all cancers occur due to abnormalities in DNA sequence the fibrin protection is a basic condition for the existence of a malignant cell and the metastasis of malignant tumor to prevent the identification by immunodefence of the body (Chin et al., 2011).

Experimental studies support the hypothesis that cancer progression and the immune system can be influenced by heparins. They can affect proliferation, migration, and invasion of cancer cells in various ways (Smorenburg and Van Noorden, 2001).

In conclusion to all these findings therapies with a fibrinolyticum (rt-PA) and heparin should be undertaken to support the therapy with tumor cell antibodies and enhance the phagocytosis of tumor cells by the immune system of the body. This fibrinolysis should be performed as after a myocardial infarction, but over a longer time period and can be applied in clinical trials after chemotherapy, alone or combined with a radiation treatment, different growth inhibitors or monoclonal antibodies.

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