RESEARCH ARTICLE

Fibrin and Extracellular Matrix: Scaffolds and Network for Malignant Cells

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Abstract:

Malignant cells build up a protective shield in form of a fibrin meshwork surrounding the tumor which helps it to escape the body's immune system. In addition, tumor and stromal cells provide with an abundant extracellular matrix (ECM) consisting of proteoglycans, collagens, glycoproteins and glucosaminoglycans an additional scaffold with the capacity to bind large quantities of immunsuppressive substances. Many investigations found that heparin has a wide variety of positive effects counteracting these shielding and immunosuppressive properties of the ECM. Heparin can bind and neutralize many protective substances produced by the tumor cells. It inhibits cross-linking of collagen by deamination, and reduces the expression of FAK, LOX, glucosamines and proteoglycans. By these actions it prevents the development of a stiff and rigid ECM which presents additionally an effective scaffold for the tumor cells and also reduce the efficiency of therapeutic methods. In an ambulant trial with exogenously-added heparin in high dosages the survival probability over three years was significantly higher than without ($p<0.001$). Therefore, a combined therapy with a fibrinolyticum and heparin should be considered. This auxiliary treatment has the potential to support established therapy and improve anti-tumor response by the immune system.

Keywords: fibrin, heparin, FAK, LOX, glucosamine, proteoglycans

Introduction:

Utilizing escape mechanisms cancer cells bypass normal proliferation controls and are able to colonize or invade other tissues. They have an altered carbohydrate metabolism with a resistance to hypoxia and an abnormal ability to survive stress and DNA damage (1, 2).

The pathogenesis of hemostatic disorders in cancer is complex and reflects the interaction of different mechanisms, i.e. activation of the coagulation endothelial factors and activation of additional components which increase rigidity and stiffness in the extracellular matrix. The clotting system and the network of different glucosamine and proteoglycans represent obviously an effective scaffold for the tumor cells. Already in 1996 we recognized a slower progression under a high dosage of heparin that was applied as a prevention against thrombosis (3, 4).

These clinical findings support the assumption that heparin has the potential to significantly influence cellular metabolism and the formation of the ECM (5). In this project we investigated the complex and effective scaffolds and protection system that malignant cells form against the immune system and explored the potential of an antitumor therapy with a combination of fibrinolytica and heparin.

Material and Methods:

Immunohistochemistry

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 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 (6).

Clinical study design

As a supplement to our immunohistochemical investigations we designed a randomized controlled trial with a high dosage of heparin (7). This study entered only patients with a radiological (MRT, CT) and histological certified carcinoma or sarcoma and in some cases one metastasis. This presented only an additional and preventive provision during the therapy according to the oncological guidelines.

In this study (1995-2018) participated 51 patients (27 males and 24 females) with an average of 50.2_years. The members of the two groups were randomized with an age of 51.1 years (heparin group) and placebo group 48,8 years in average. In the placebo group there was an incidence of 8 thrombosis and cured with 20 or 40 mg enoxaparin for about one week according to the guidelines. In the heparin group (13 males, 11 females) the participants got a complex information about efficacy and side effects of this high dosage heparin therapy before they entered the investigation. For three years the patients of the heparin group got four times a week 25,000 IE heparin intravenous and during the rest of the week 80mg (8,000 IE) subcutaneously enoxaparin-natrium one time daily. The participant patients had to fill out a monthly protocol about their heparin therapy (UFH, LFH). Platelets were controlled monthly. No thrombopenia or HIT had been registered. The different tumor types of all patient consisted of: 13 mamma-Ca, 15 prostate-Ca, 3 pancreas-Ca, 6 stomach-Ca, 9 colon-Ca, 2 fibrosarcoma, 2 acute

myeloblastic leukemia and 1 acute lymphatic leukemia.

The distribution of the tumor types in the heparin- and control group was again nearly proportional with: 6 mamma-Ca, 7 prostate-Ca, 2 pancreas-Ca, 3 stomach-Ca, 4 colon-Ca, 1 fibrosarcoma and 1 myeloblastic leukemia in the heparin-group and 7 mamma-Ca, 8 prostate-Ca, 1 pancreas-Ca, 3 stomach-Ca, 5 colon-Ca, 1 fibrosarcoma, 1 acute myeloblastic leukemia and 1 acute lymphatic leukemia in the control group.

Results

We tested archival tissue sections of various malignancies for the expression of fibrin by immunohistochemistry (IHC). In all tumors fibrin expression was detectable (Fig. 1). Interestingly, the malignant cells were identified as the source of fibrin. The stromal cells were largely negative for fibrin. Cultured tumor cells also expressed fibrin (Fig. 2). Thus, the malignant cells, independently of ectodermal or mesenchymal derivation are the origin of hypercoagulability and fibrinolytic system inhibition.

Figure 1. Fibrin is expressed on tumor cells of different carcinomas
A Pancreatic carcinoma (isotype control x 20, immunohistochemistry)
B Ovarial carcinoma (isotype control x 20, immunohistochemistry)
C Breast carcinoma (isotype control x 20, immunohistochemistry)
D Colon carcinoma (isotype control x 20, immunohistochemistry)
Slides were counterstained by hematoxylin and eosin, x20.

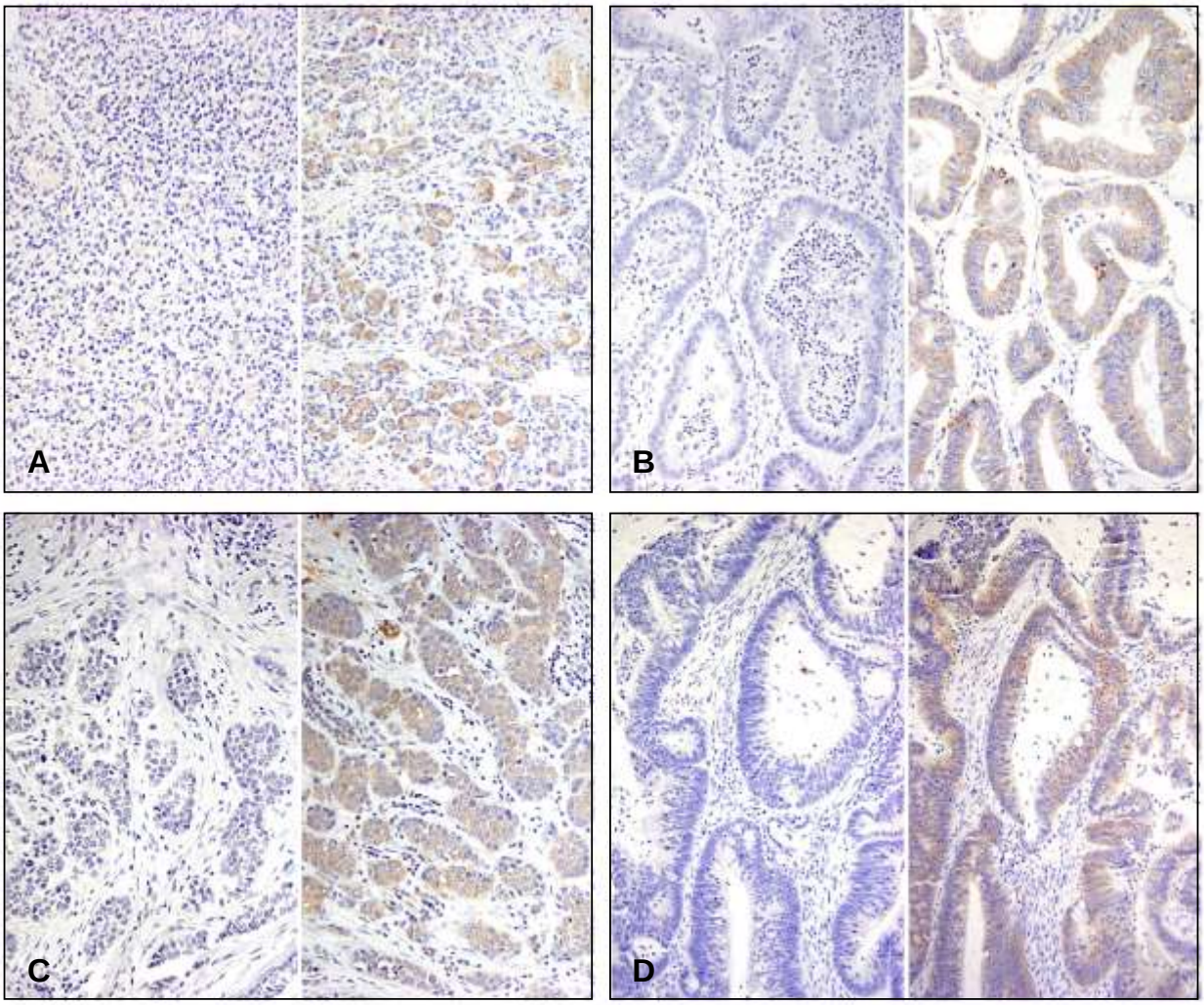
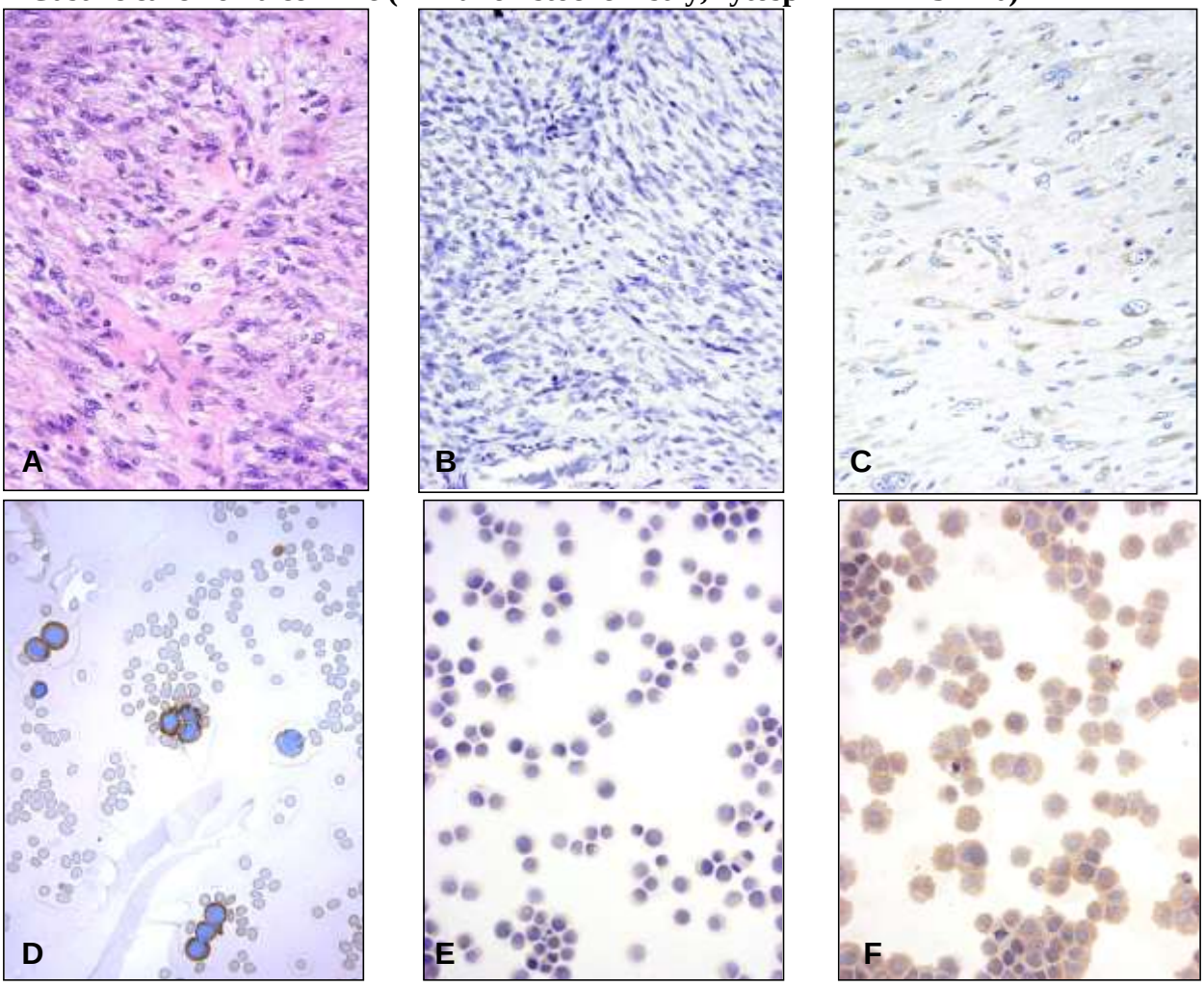
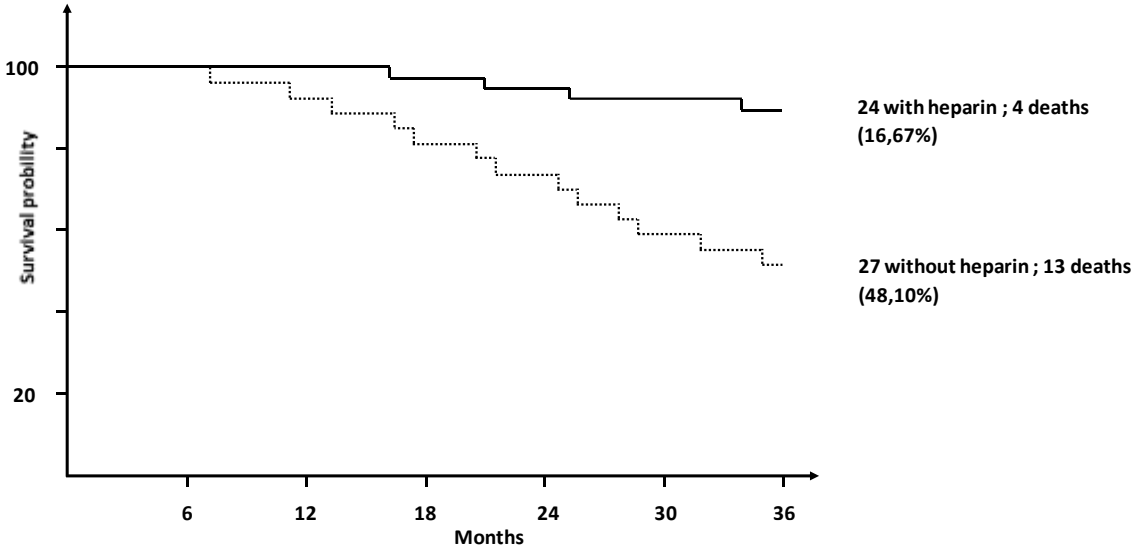


Figure 2. Fibrin was also expressed on tumor cells of
A Leiomyosarcoma (HE x 20)
B Leiomyosarcoma (isotype control x 20)
C Leiomyosarcoma (immunohistochemistry x 20)
D Acute myeloblastic cells HMO2 (immunohistochemistry x 40)
E Gastric carcinoma cell line (isotype control, zytospin MKN 45 x 40)
F Gastric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



In the ambulant trial we tested the possibility of a therapy with exogenously-added heparin. Patients received i.v. heparin (25,000 IU) four times a week and during the other days 80 mg enoxaparin s.c. for three years. The survival probability in the treatment group over three years was significant higher ($p < 0{,}001$) than in the control group (Fig. 3). Observed bleeding complications were low grade (WHO grade I: mouth, nose, injection mark, no gastrointestinal) without the need of pharmacological or surgical treatment. The incidence of bleeding complications was in the heparin group 5,9 % and in the placebo group 1,8 %. The data were sampled between 1995 and 2018 in the ambulance with written consent of the patients.

Fig. 3: Survival probability of 51 randomized patients with heparin and different malignant diseases (p < 0,001) and without heparin during the oncological therapy according to the guidelines over three years. (Kaplan-Meier-Plot)



Discussion

Almost 90% of new drugs entered into clinical development on promising preclinical findings fail to yield sufficient efficacy and safety to receive FDA license (8). Such high rates of discordance between experimental investigations, preclinical results and effects with cancer patients have prompted mounting theories that cancer cell developed escape mechanisms and a protective ECM barrier, that are effective against both, the immune system of the body and the antitumor therapy. Moreover, tumors produce factors and components of the coagulation system e.g. factor VII, VIII, IX, X, XII, XIIIa and prothrombin (9,10). These factors are obviously produced together with fibrinogen and fibrin in the malignant cells deposit fibrin around the malignant cells as a barrier and shelter against the immune system (4, 6).
That these fibrinoid depositions can play an important role in immune escape, is demonstrated by the fact that the intrauterine perivillous fibrinoid contains PD-1 to protect fetus and mother against immunoreactions of each other. It plays also an essential role in the human placenta with the functional relevance of a scaffold for the morula in the mucosa because it protects the new tissue against the cellular immune system of the mother (11).

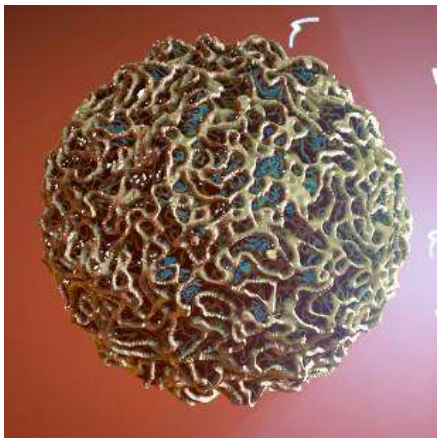
It is evident that the urokinase plasminogen activator system plays an essential role as fibrinolysokinase in tissues with malignant cells (6, 12).
Urokinase was originally isolated from human urine and is also present in the blood and extracellular matrix of many tissues. It activates plasminogen to plasmin by triggering a proteolytic cascade. It is supposed that it is produced in the iuxta-glomeral tissue and in tissues where urokinase occurs in higher concentrations i.e., renal pelvis and ureter malignant tumors are very seldom. Therefore, it should be a consequence of therapy to use urokinase or the improved rtPA (12).

Experiments in our laboratory with tumor cell lines *in vitro* showed in two experiments no influence of heparin on proliferation or survival. Therefore, it is plausible that heparin exerts its effect mainly on the tumor microenvironment and its ECM (13). Own investigations with heparin supplement of the therapy over three years shows a clear survival benefit of this supplementation.

Heparin and heparan sulfate are linear polysaccharides composed of repeating N-acetyl glucosamine-glucuronid as disaccharide units. Heparan sulfate differs from heparin in that it is less sulfated. They are found attached to case proteins in proteoglycan on cell surfaces and within the extracellular matrix of nearly all mammalian cells and tissues predominantly mast cells, liver and small intestine. While heparin has no fibrinolysis properties like tissue plasminogen activator or urokinase, it allows the body's natural clot lysis mechanism to take place. Additionally, heparin has antiphlogistic properties and is therefore an ideal supplement to rtPA. With 3 sulfate ester/amide sulfate and a carboxyl group per disaccharide unit it has the highest negative charge density of any known biological molecules. This contributes to its very strong electrostatic interaction with thrombin, a lot of glucosamines, glucosaminoglycans and signaling proteins (14). Previously, it was demonstrated that heparin inhibits progression and migration of cancer cells. In contrast to fondaparinux the concentrated negative charge density of heparin attenuates metastasis in a L-and P-selectin, integrin VLA-4 and syndecans binding manner (15). Heparin is also found intracellularly, where it stimulates DNA synthesis in normal cells and inhibits it in tumor cells (16). The high production and concentration of heparin might be one reason that in the small intestines malignant tumors and metastases are very rare. Moreover, malignant cells activate in response to intracellular heparin uptake a signaling cascade, which inhibits adhesion and migration of cancer cells and affects thereby cancer progression. Additionally, heparin stimulates the expression of p53 in malignant cells and its accumulation in the nucleus. This results in a decrease in FAK promoter activation and explains the reduced FAK transcript and protein levels. Thus, heparin acts in the regulation of malignant cell adhesion and migration by influencing a p53/FAK signaling pathway (17).

DNA methylation a widespread process and occurs already during the embryogenesis by demethylation and remethylation (18). Although, DNA methylation is maintained by methyltransferases and induced by genetic mechanisms, CpG island methylations and epigenetic or histone modifications sulfotransferases which participates in sulfate biosynthesis and glycan modification are also essential for its regulation (19, 20).

Fig. 4: Malignant cell with a tight meshwork of fibrin. To provide and secure the complex and intensive metabolism of a malignant cell the fibrin chains are permeable for the substances of metabolism but also as a protection against cells of the immune system.

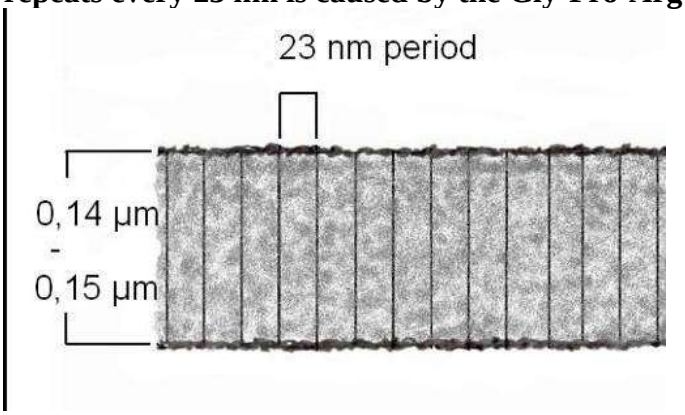


Tumor cells with mutated DNA (wt DNA) in comparison to the normal cell form a fibrin scaffold (Fig. 4). Already cells which are exposed to chronic inflammation, physical or chemical irritation show damages of DNA and are reversible by a repair machinery after ceasing their negative influence. Reduced DNA repair in the presence of increased DNA damage increases carcinogenic mutations (21). Therefore, tumor induction is a complex process with numerous modulatory factors that determine the individual`s probability and heparin can decrease it by increasing the immune surveillance (22). On average 15 driver and 60 passenger mutations are found in cancer cells (23). Simultaneously alterations in p53 are seen mostly connected with a shift to the glycolytic pathway. Especially hypoxia stimulates a complex signaling network in cancer cells including HIF, PI3K, MAPK and NFkB pathways and can cause genomic changes i.e. p53 (24).

The activation of the telomeres DNA damage response will define the fate of cells according to the functionality of cell cycle checkpoints. Therefore, dysfunctional telomeres can suppress cancer development by engaging apoptotic pathways, but they can also promote tumor initiation. In this case heparin inhibits not only reverse transcriptases but also telomerase and stimulates telomeres (25).

In the ECM heparin disrupts signaling complexes and serves as a ligand sink for pathways regulating tumor proliferation, angiogenesis, differentiation and migration. The two known human sulfatases 1 and 2 are soluble enzymes that catalyze the cleavage of the 6-O sulfate ester bond. Whereas sulfatase 1 suppresses tumor cell proliferation and invasion, sulfatases 2 enhance these processes (26). These findings show that the metabolism of the cell metabolizes heparin according to the requirement. Therefore, only an excess application of heparin has a therapeutic effect, which explains why the endogenous heparin produced by the body, predominantly in the mast cells does not induce the same effects as the therapeutically applied heparin (27). These results are also supported by up-regulation of sulfatase 2 in human hepatocellular and breast carcinoma which deactivated heparin sulfate and opposite effects were observed in sulfate 2 – knockdown models (28). However, heparin as inhibitor of heparanase is sensitizing activated lymphocytes which can penetrate the vascular endothelium and infiltrate target tissues (29).

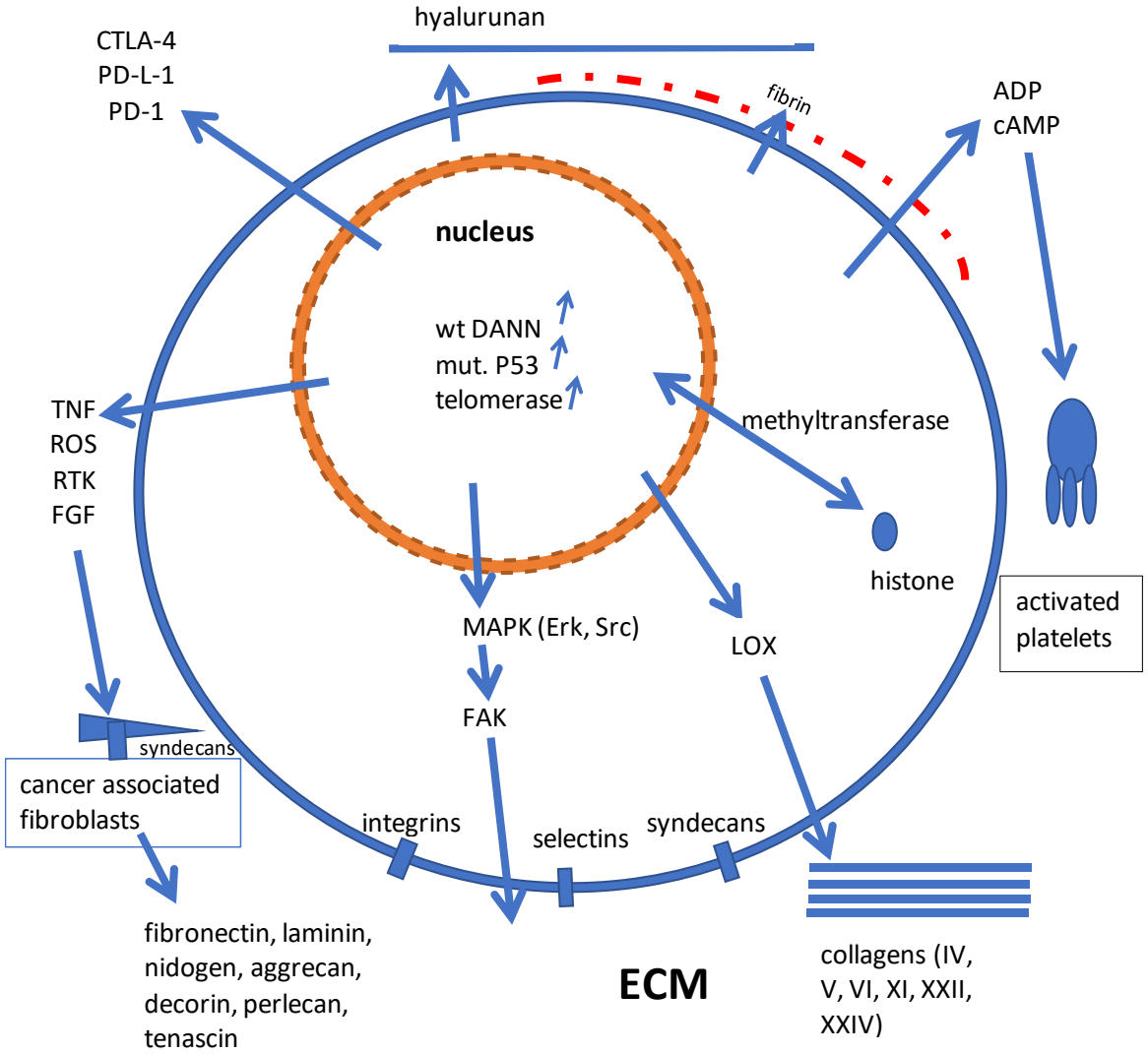
Fig. 5: The electron micrograph of fibrin shows a 23 nm period along the fiber axis and is half the length of a fibrinogen molecule. By this units the breadth of a fibrin protofibril can be calculated for 0,13-0,15 µm (Lit. Stryer p. 305, Fig. 10.29). The periodic structure that repeats every 23 nm is caused by the Gly-Pro-Arg sequences (Woodhead JL, Slayter H).



Tumors activate various mechanisms to inhibit immune responses. These include stimulation of regulatory T cells and dendritic cells (30). Escape mechanisms such as PD-1/PD-L1,checkpoint activation, immune suppressive cytokines like IL10 and TGFß. LAG-3, TIM-3, VISTA, CD244, CD160 and BTLA can act to fine-tune the cellular fate tumor infiltrating T-cells (31). Heparin also plays an active role in immune escape: it binds to selectin and integrin, blocks the formation of cytokine complex signal pathways and interrupts the adhesion of cancer cells by decreasing the expression of E-cadherin and catenin (32, 33).

The tumor microenvironment comprises the ECM, the vasculature and various tumor associated cells of the immune system. It varies strongly in composition, density and function to the microenvironment in normal tissues. But there are also strong differences between various tumors concerning ECM rigidity, dysfunctionality of the vasculature or composition of the collagen matrix and proteoglycan content. Chemoresistant tumors are characterized by increased expression of collagens and collagen-stabilizing enzymes such as lysyl oxidase (LOX). Similarly, the high-water content of hyaluronic-rich pancreas tumors, sarcomas or glioblastoma creates a high interstitial pressure, interfering with drug distribution (34). Finally, the therapy with therapeutic antibodies is strongly restricted by the rigid ECM.

Fig. 6: Tumor cells within a meshwork of fibrin, activated platelets, cancer associated fibroblasts and extracellular matrix with different glycoproteins and proteoglycans (schematically). These substances form by tight connections (FAK, LOX) together with the fibrin meshwork an additional scaffold for malignant cells in the extracellular matrix (ECM). This causes an increase of rigidity and stiffness in the extracellular matrix.



Heparin shares a similar function with hyaluronate and other glycosaminoglycans. Heparin stored in mast cells prevents hyaluronan synthesis in intracellular compartments and subsequent autophagy. This suggests also a new role for endosomal heparinase in these organs and especially in combination with diabetes (35). The addition of recombinant fibroblast growth factor-2 resulted in the rapid binding to heparin as a hyaluronate-heparin conjugate (36). There are synthesized mainly in the mucosa cells of the small bowel and liver in a non-sulfated form which is the acetylated and sulfated sequence. Some of these variously sulfated sequences act as anticoagulants by binding specifically to antithrombin which accelerates its sequestration of thrombin. Hyaluronate (also called hyaluronic acid or hyaluronan) obviously plays a central role in the ECM of tumors. At least one of the sugar

molecules in the repeating unit has a negatively charged carboxylate group, which is usually attached and linked to proteins to form proteoglycans and can bind to a wide range like chondroitin sulfate, dermatan sulfate aggrecan and keratin sulfate and affects their activity.
A link protein binds both, the core protein of a proteoglycan and the hyaluronan chain thereby stabilizing the aggregate. Heparin and heparan sulfate with the highly negative charge given in a high dosage are therefore very effective in constructing a soluble compound preventing increased rigidity in the ECM (36). Also a downregulation of cAMP induced by opioid receptor activation using D,L-methadon kills and sensitizes leukemia cells for doxorubicin treatment (37). Since cancer cells produce encephalin, they control with this endogenous opoid system not only cell proliferation but also activate a pain-modulating system (38,39).

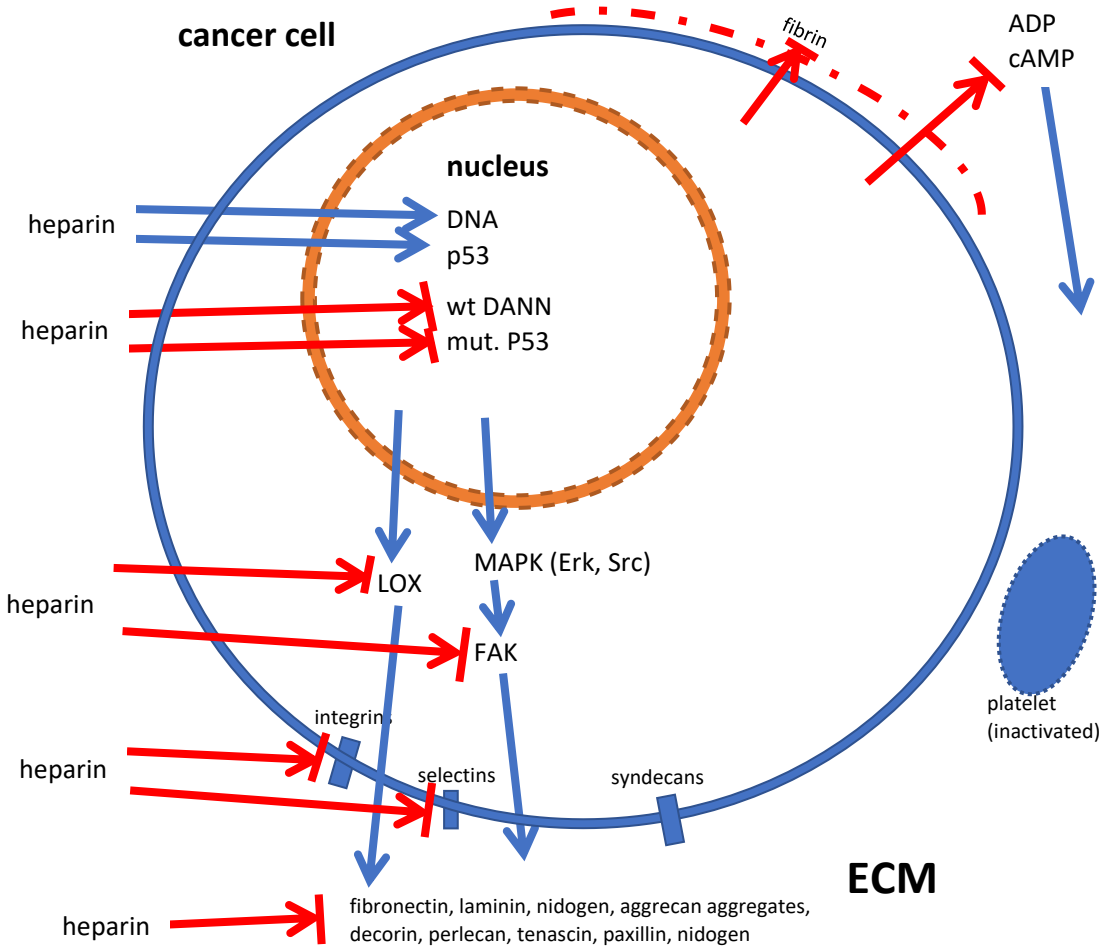
Heparin inhibits LOX (lysyloxidases) by deamination, makes a reduced downregulation of p53/FAK, integrins, segretins, laminin, hyaluronate, collagens 1 and fibronectin and binds by its negative charge density insolubly hyaluronate (40). By these reactions it prevents in the extracellular matrix the development of stiff and rigid substances which presents additionally an effective scaffold for the tumor cells against the immune system of the body and reduces also the efficiency of therapeutic methods.

Tumors with high LOX activity and collagen content are significantly stiffer and more rigid than normal tissues, as shown by strain rotational rheometry (41). Furthermore, the clustering of integrins induced by tissue stiffness or by myosin-induced contractility promotes malignant behavior by disrupting adherend junctions and enhancing growth (40). These ECM changes caused by malignant cells are the reason that tumors can be visualized in MRT, PET and sonography (42).

Additionally, heparin functions in the ECM catalytically to modulate vascular endothelial growth factor (VEGF) binding site availability on fibronectin. Cryptic VEGF bound to fibronectin remains available after heparin was removed (43). Besides these findings other authors demonstrated that multiple isoforms of laminin as a key component of the basement membrane ECM regulates tissue morphogenesis are bound through their heparin binding domains (HBDs) (43, 44).

Tumor cells under hypoxia are producing cluster of integrin, hyaluronate, aggrecan aggregates, fibronectin or collagen to induce an increase of stiffness and rigidity of the tissue (45). Especially, increased collagen production and increase of LOX activity are accompanied by bad prognoses (46).
Other malignant tumors are sensitive to increase of FAK (focal adhesions kinase), ERK or Src signaling, the production of paxillin, laminin, integrins and selectins. These cues stimulate migration, invasion and metastasis in these tumors. Heparin regulates p53/FAK dependent signaling in adhesion and migration and can be used to down-regulate FAK.

Fig. 7 Heparin stimulates (→) DNA, p53 and inhibits (→|) the production of mutated or wild type (wt) DNA, mutated p53 and different glycaminoglycans, proteoglycans, collagens and glycoproteins depending on the applied dosage. Thereby the formation of scaffolds for malignant cells in ECM are reduced.



Heparin inhibits proliferation and migration of tumor cells. The different phases of metastasis are detachment, invasion mostly in lymphatic or venous vessels, extravasation and attachment in distant organs, often described as lymphangiosis carcinomatosis (not lymphangitis) in lymph nodes, pleura and lungs. The fibrin meshwork is a presupposition by inactivating or degrading of the tight junctions and the defacement of the malignant cells in the tissues (47). During the intravascular distribution the malignant cells are protected by a fibrin meshwork (Fig.: 2D). Therefore, fibrin is not only a passive scaffold for the malignant cell but also induces the detachment from the tumor tissue. Stromal fibroblasts facilitate this detachment by an invadopodia-independent matrix degradation process through GTPase, Cdc42 and metalloproteases. The attachment of malignant cells in the distant tissue is promoted mostly by activated platelets, FAK, vitronectin, fibronectin and thrombospondin. Heparin as previously mentioned is inhibiting signaling cascades (TGFß, ROS, RTKs, TNF, FGFs) in cancer associated fibroblasts and activated platelets (47). The release of platelet-derived chemokines CXCL5 and

CXCL7 depends on both, a thrombin-mediated activation and a direct interaction between tumor cells and platelets. However, heparin is able to reduce these chemokines release by thrombin inhibition (48). There is also a feedback or mutual influence between activated platelets, collagens, glycoprotein VI, fibronectin, factor V, fibrinogen, ADP and Ca^{2+} inducement by malignant cells. Especially astrocytoma and glioblastoma are spreading out fast in brain because they are using hyaluronates or collagens as tracks for migration and metastasis (49).

As a summary, our results and the scientific literature show that by methylation the mutated DNA (wt DNA) of the malignant cells regulates the construction of the protective scaffolds. With our immunohistological method we were able to demonstrate that all malignant cells build up a scaffold of fibrin against the immune system of the body. Moreover, the increase of FAK and LOX by phosphorylation as described previously forms a further ECM barrier which can be reduced by heparin as shown in our randomized clinical trial. Heparin downregulates p53/FAK, inhibits LOX-catalyzed crosslinking by deamination, reduces the production of laminin, paxillin, perlecan, tenascin, collagens and fibronectin and binds by its negative charge density insolubly hyaluronate and dermatan (Fig.6, 7).
By these actions it prevents further stiffening of the ECM which would additionally increase the protection against the immune system and reduce the efficiency of therapeutic intervention (50). The mutated DNA of malignant cells induces obviously these escape mechanisms. Therefore, heparin is an excellent supplement to a fibrinolyticum in prevention and therapy. It has additionally the advantage that there is an effective antidot with protaminsulfat to avoid bleeding complications.

In conclusion to all these findings therapies with a fibrinolyticum (rt-PA) and high-dosage heparin should be considered to support therapy with cytotoxic agents, targeted therapies or antibodies and to enhance the efficacy of the immune system in fighting the tumor. This fibrinolysis should be performed similar to its employment in myocardial infarction therapy, but over a longer time period. It can be applied alone or combined with radiation therapy, different growth inhibitors or monoclonal antibodies. Moreover, an improvement of the efficacy of chemotherapy during an antitumor treatment can also be expected.

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