Mechanism of collagen synthesis in Experimental carcinoma in αvβ3 integrin knockout mice.

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CONTENTS

Abstract...............................................................................................................................................2

1. Introduction.....................................................................................................................................3
1.1. Tumor microenvironment.................................................................................................3
1.2. Collagen....................................................................................................................................3
1.3. Biosynthesis of collagen.................................................................................................4
1.4. Collagen in tumor tissue.................................................................................................5

2. Methods and Materials..............................................................................................................7
2.1 Ex vivo system for collagen synthesis................................................................................7
2.2 Immunoprecipitation of collagen....................................................................................8
2.3 Immunoprecipitation of denatured collagen....................................................................8
2.4 Immunoprecipitation of CT26 tumor samples......................................................................8
2.5 Extraction collagen from CT 26 culture.............................................................................9
2.6 Collagen extraction from CT26 tumors.............................................................................10
2.7 Western blotting....................................................................................................................11
2.8 PCR genotyping...................................................................................................................11

3. Results and Discussion.............................................................................................................13

4. Conclusion...................................................................................................................................23

5. Future projects...........................................................................................................................23

4. Acknowledgement....................................................................................................................24

5. References..................................................................................................................................25-27
Abstract

The tumor microenvironment plays a major role in the progression of cancer and has attracted an increased interest in recent years. Due to the high interstitial pressure that characterizes carcinoma bioavailability of anti-cancer drug to the cancer tissue is reduced, which is a major obstacle in chemotherapy of cancer. Little is known about how malignant cell controls the collagen synthesis and degradation in the tumor microenvironment. Moreover in recent years interest of collagen in tumor microenvironment has increased due to the fact that novel markers such as tumor associated collagen signature (TACS) are being used to characterize tumors. Understanding of tumor microenvironment has given a hope for new therapies in combating cancer. In the present project collagen in particular type I which is abundant in animal kingdom has been studied in beta 3 integrin null mice with emphasis on the mechanism of collagen synthesis in experimental carcinoma. This present study also aimed at finding the effect of GLEEVEC® (imatinib mesylate) on collagen turn over studies in experimental carcinoma. This study shows collagen turn over was higher in integrin β3 knockout mice than wild type, as band intensity of α1 and α2 were higher in tumors grown in beta 3 integrin deficient mice and treatment of tumors with Gleevec did not show any major change in α1 and α2. This study also shows cancer CT26 cells also produce collagen Type I in in vitro culture.
1. Introduction

1.1 Tumor microenvironment

A solid tumor mass consist of malignant cells, fibroblasts, infiltrated inflammatory cells, endothelial cells and pericytes (1). A better understanding of tumor microenvironment will help in elucidating the host interactions in cancer growth and metastasis. In tumor microenvironment fibroblast are the main source of collagen type I which forms the major component of the Extracellular Matrix (ECM) (2). The tumor vasculature has also been shown to be abnormal with leaky tortuous, unevenly distributed, chaotic and highly permeable blood vessels. At the tumor–host interface tumor vasculature density was found to be greater than at central zone (3). Structurally the interstitium in the tumors is different to the interstitial space in the normal tissue with high collagen content, low proteoglycan and hyaluronan concentrations, and absence of a lymphatic network. These differences may explain the pathologically high interstitial fluid pressure and reduced bulk fluid flow (4, 5).

Fibroblasts in the stroma are important sources of matrix metalloproteinase (MMPs) and collagen (1). Desmoplastic stroma with tumor fibroblasts has been evidenced to play a role in contractility of tumor cells inducing tension dependent ECM remodeling which further promotes reorientation of collagen fibrils radially in the invasive front of tumor (6). Tumor invasion and metastasis is controlled by cytokines, growth factors, integrins signaling and matrix metalloproteinases. Secretion and activity of MMP induces global reorganisation of collagen fibers has been shown by culturing the tumor explants in three dimensional collagen gels used as a model for the invasion process in desmoplastic stroma (7).

1.2 Collagen

Collagen provides the tissues with mechanical strength and constitutes the major scaffold for cell attachment and anchorage of extracellular matrix macromolecules. Collagens share the common structure motif of three polypeptide chains intertwined in a triple
helical configuration as shown in Fig 1. Collagen type I consists of two collagen α1 [I] and one collagen of α2 [I] chain. Fibrillar collagens I, II, III, V, and XI all assemble to form ordered fibers through hydrophobic and electrostatic interactions between adjacent chains (8). Several extracellular matrix (ECM) proteins such as fibronectin, fibromodulin and decorin bind to type I collagen (9, 10). Collagen type IV forms the basement membrane. Type I collagen interacts with cells such as fibroblasts, and during blood clotting with platelets. Collagen in its native form binds to several β1 integrins such as α1β1, α2β1, α10β1, α11β1. Indirect binding to collagen fibres has also been reported via αiβ3 through bridging by fibronectin (11, 17). The importance of type I collagen for many human diseases can be exemplified by fibrosis, cancer and atherosclerosis.

1.3 Biosynthesis of collagen type I

Collagen type I is synthesized as a procollagen molecule with propeptide at its N- and C-terminal ends. Collagen synthesis involves post-translational modifications and final incorporation into fibril structures. The main intracellular steps in the assembly of a procollagen molecule involves cleavage of the signal peptides, hydroxylation of certain proline residues to 4-hydroxyprolin and 3-hydroxyproline which are required for the stabilizing the folded structure (12). Triple-helix formation is initiated after synthesis of the procollagen polypeptide chains is completed and the chains associate to form interchain disulfide bonds within the C-propeptide as shown in Fig 1. From this single nucleation site at the C-terminus triple-helix formation moves toward the N terminus of the molecule in a zipper-like fashion controlling the rate of triple-helix formation and prevent misfolding of procollagen molecules. The procollagen molecules are transported from the Endoplasmic Reticulum (ER) through the Golgi stacks without leaving the lumen of the Golgi cisternae. The extracellular steps in biosynthesis include enzymatic cleavage of the N and C propeptides, self-assembly of the collagen molecules into fibrils by nucleation and propagation and formation of covalent cross-links. The molecular chaperone, Hsp47 plays an important role in collagen synthesis by binding to regions of
the triple helix that are of low stability and thus helps to stabilize the molecules within the ER (13). Homozygous knock-out of Hsp47 in mice leading to embryonic lethality indirectly shows its requirement collagen synthesis during normal development (14).

### 1.4 Collagen in Tumor tissue

Low efficacy of conventional anticancer therapies has underlined the phenomenon of physiological resistance by tumors owing to poor uptake of drugs in the carcinoma tissue. Tissue resistance towards diffusion of macromolecules has been shown to depend on glycosaminoglycan and the collagen component of the ECM. Furthermore the diffusion of IgG increases by treatment of tumors with collagenase (15).

In recent years interest of collagen in tumor microenvironment has increased due to the fact that novel markers such as tumor associated collagen signature (TACS) are being used to characterize tumors (7). Molecular diffusion for large molecules is impeded by collagen at physiological concentrations as has been shown from collagen gels made from commercially available collagen (vitrogen) in vitro. Diffusion in pure collagen gels has shown to mimic that in the tumor interstitial matrix over a wide range of particle sizes (16). Tumor-associated collagen signatures are shown to be useful as pathological markers to monitor physiological changes in tissues in cancer and its progression (7).

Proteolytic remodeling of collagen network in the ECM may be the general features of tumors during tumor angiogenesis growth, invasion, and metastasis process. Molecular defects in the assembly process may be associated with the loss of normal cellular growth regulation mechanisms. In a recent report which implicates the role of lysyl –oxidase-mediated collagen cross linking causes tumor matrix stiffening, further increases the integrin signaling and invasion process in tumors (18). Experimental carcinoma in animals treated with GLEEVEC (STI571), formerly known as CGP57148B, a selective PDGF receptor kinase inhibitor exhibit increased capillary to interstitial transport of $^{51}$Cr-EDTA in rat colonic carcinoma and decreased interstitial fluid pressure (19). In this study Gleevec was used to investigate any alteration in collagen synthesis mechanism. This study also investigates the role of Integrin $\alpha v \beta 3$ in collagen turnover.
Figure 1. Biosynthetic pathway of collagen synthesis and assembly process.
2. Materials and Methods

2.1 Ex vivo system for collagen synthesis

The present study used an Ex vivo system for collagen synthesis. Colon tumors from 3 (Itgb3 -/-, vehicle) + 3 (Itgb3 -/-, vehicle) mice were dissected to >300 µm sections using a Vibratome and weighed in prepared sterilized 1.5 ml eppendorf tubes containing approximately 1.3 ml of PBS to get a tissue weight nearest to 160 mg with 2 samples per tumor. Tumor explants were transferred into a 24-well plate containing 0.5 ml proline–glycine free Eagles’ Minimal Essential Media (EMEM) (Gibco 42360-024) supplemented with 0.284 mM ascorbic acid, 1 µM FeSO₄, 10 µM GM 6001, 1 mg/ml BSA (Roche Diagnostics), 50 µCi/ml ¹⁴C-proline and ¹⁴C-Glycine, 1:6 ratio (PerkinElmer) and were incubated for 6 hours in a humidified atmosphere comprising 95% air and 5% CO₂ at 37 °C. After 6 hours of Exvivo incubation samples were transferred to prepared 1.5 ml eppendorf tubes, freeze dried for 40 hours and weight was recorded. Other tumor explants were processed for collagen extraction by adding 1.2 ml of 0.5M acetic acid containing 3mg/ml pepsin (Sigma) and incubated for 1 hour and the pH was adjusted to 2.5 and incubation step was carried at 4 °C for 23 hours and later centrifuge at 13000 rpm for 30 minutes. Supernatant was transferred to a 2ml Eppendorf tube and remaining tissue was stored at 20 °C. To the 1.5 ml supernatant fraction 500 µl of 2.8 M NaCl was added to give a final concentration to 0.7M NaCl to precipitate collagen and incubated for 24 hours at 4 °C. The supernatant samples were centrifuged at 13000 rpm for 30 mins (Biofuge 204631) and supernatant fraction was discarded. The pellet was washed twice in 0.7 M NaCl in 0.5 M Acetic acid and centrifuged for 13000 for 30 mins (Biofuge 204631) between each wash.

To the other tumor tissue sample 1 ml of 0.5 M Acetic acid was added followed by incubation for 1 hour at room temperature. The pH was adjusted with acetic acid or 6 M HCl sample was vortexed extensively and incubation is continued for another 23 hours at room temperature. Further pH was adjusted to 7.4 using Tris base Pellet from the
resultant centrifugation was redissolved in 50 µl 2XSB (0.2M Tris –HCl pH 8.8 buffer, 18% glycerol, 0.01% Bromophenol blue) and vortexed and heated at 95 °C for 10 mins before loading on SDS Polyacrylamide gel. SDS/PAGE was performed by loading equal volume from the samples on a 6% polyacrylamide gel run at 60 volts initially and further increased to 100 volts. Gel fixation was performed using 50% methanol, 45% water. 10% Acetic acid for 30 mins and stained with 0, 1% Coomassie for more than an hour, followed by gel destaining in water and was scanning. The dried gel was exposed on an imaging plate for 24 hours depending on the initial development and the band intensities on the imaging plate was scanned with a using BAS-2500 (Pharos Fx™ molecular imager).

2.2 Immunoprecipitation of collagen
Collagen at a concentration of 200 µg/ml dissolved in (1x) PBS solution was taken in equal volumes (1ml) in four 1.5ml eppendorf tubes. To the tubes varying concentration from 75 µg, 100 µg, 150 µg of P13 antibody (polyclonal antibody were raised towards CNBr digest of collagen type I) was added to each of the tubes with a blank tube without P13 antibody. Tubes were incubated at 4 °C for 5 hours with P13 antibody. Then 100 µl of Sepharose activated protein-A was added each of tubes and incubation was carried further at 4 °C for 2 hours. Tubes were centrifuged at 14000g for 3 mins, resultant supernatant was stored. Pellet was then washed with 1x PBS twice. Supematant from the centrifugation was discarded. To the pellet fraction 25µl of (2X) SB (0.2M Tris –HCl pH 8.8 buffer, 18% glycerol, 0.01% Bromophenol blue) buffer is added and heated for 95 °C for 10 mins and spun down and above supernatant is loading on SDS polyacrylamide gel. SDS/PAGE was performed by loading equal volume from the samples on a 6% polyacrylamide gel run at 60 volts initially and later increased to 100 volts.

2.3 Immunoprecipitation of denatured collagen
Collagen of concentration 200 g/ml dissolved in (1x) PBS solution is denatured at 55 °C for 20 mins volumes (1ml) in two 1.5 ml eppendorf tubes. To the tubes varying concentration from 100 µg of P13 antibody (polyclonal antibody were raised towards CNBr digest of collagen type I) was added to each of the tubes. Tubes were incubated at
room temperature for 5 hours with P13 antibody. Then 50 µl of Sepharose protein-A was added to each of the tubes and incubation was carried at room temperature for 2 hours. Tubes were centrifuged at 14000g for 3 min (Biofuge 75003235), resultant supernatant is stored. Pellet is then washed with 1x PBS twice. Supernatant from the centrifugation was discarded. To the pellet fraction 25 µl of 2XSB buffer (0.2M Tris –HCl pH 8.8 buffer, 18 % glycerol, 0.01%. Bromophenol blue) is added and heated for 95 °C for 10 mins and spun down and above supernatant was loading on SDS polyacrylamide gel. SDS/PAGE was performed by loading equal volume from the samples on a 6% polyacrylamide gel run at 60 volts initially and further increased to 100 volts.

2.4 Immunoprecipitation of CT26 tumor samples
Collagen extracted tumor samples were Immunoprecipitated after pH adjusted to 7 later 100 µg of P13 antibody (polyclonal antibody were raised towards CNBr digest of collagen type I) was added and tubes were incubated at room temperature for 5 hours with P13 antibody. Then 100 µl of Sepharose protein-A is added each of the tubes and incubation was carried further at room temperature for 2 hours. Tubes were centrifuged at 14000g for 3 min (Biofuge 75003235), resultant supernatant is stored. Pellet is then washed with 1x PBS twice. Supernatant from the centrifugation was discarded. To the pellet fraction 25µl of 2XSB buffer (0.2M Tris –HCl pH 8.8 buffer, 18 % glycerol, 0.01%. Bromophenol blue) buffer is added and heated for 95 °C for 10 mins and spun down and above supernatant is loading on SDS polyacrylamide gel. SDS/PAGE was performed by loading equal volume from the samples on a 6% polyacrylamide gel run at 60 volts initially and further increased to 100 volts.

2.5 Extraction collagen from CT 26 culture
CT 26 tumor cells were cultured in T-75 flask containing RPIM media (GIBCO) supplemented with 10% bovine serum (Saveen Werner Lot No. 5795 ).Day 3 media from the t-flask was changed to RPIM (GIBCO) supplemented with 1 % fetal bovine serum (Saveen Werner Lot No. 5795) and vitamin C. cells are maintained to reach the confluency, extraction of collagen from culture is made by adding 0.5M HAC (15) ml.
crystalline pepsin (Sigma Aldrich) is added to get a final conc of 6 mg/ml, incubated for 24 hrs in 4 °C. Acetic acid - pepsin solution from T flask is spun down 10 min at 13000 rpm and supernatant is collected for collagen extraction. collagen precipitation is done by adding NaCl to make final concentration to 0.7 M to the above supernatant and left at for 24 hrs in 4°C. Supernatant is further distributed to small 1.5 ml Eppendorf tubes centrifuged at 13000 rpm for 30 min and pellet fraction is washed twice with 0.7M NaCl solution.further pellet is dissolved and pooled in 50 µL 2XSB sample buffer (0,2M Tris – HCl pH 8.8 buffer, 18 % glycerol, 0,01%. Bromophenol blue).

2.6 Collagen extraction from CT26 tumors

CT26 colon tumor tissue was weighed to nearest to 200 mg (wet weight) in two (A and B) in pretared sterilized 1.5 ml eppendorf tubes and freeze dried for 24 hours. To tube A 1 ml of 0,5 M acetic acid of pH 2.5 is added and pH adjusted to 2.5 with 6M HCl, incubated at 4 °C for 24 hrs. Later centrifuged at 13000 rpm for 30 minutes. Supernatant was transferred to a 1.5ml Eppendorf tube and remaining tissue was stored at 20 °C. Similarly to tube B 1ml of 0.5M acetic acid containing 3 mg/ml pepsin (Sigma) of pH 2.5 is added and with pH adjusted with 3M HCl incubated at 4°C for 24 hrs. Later centrifuged at 13000 rpm for 30 minutes. Supernatant was transferred to a 1.5ml Eppendorf tube and remaining tissue was stored at 20 °C. To the Supernatant from A and B fraction 0,375 ml of 2.8 M NaCl in 0.5M acetic acid is added and incubated at 4°C. Supernatant fractions from both A and B were centrifuged at 13000 rpm(Biofuge 204631) for 30 minutes and resultant pellet fraction is washed with 0,7M NaCl in 0,5M acetic acid. Pellet fraction was dissolved in 50 µl (2X) sample buffer (0,2M Tris –HCl pH 8.8 buffer,18 % glycerol, 0.01%. Bromophenol blue) and loaded on to 6 % SDS –PAGE and run at 60 Volts initially and increased to 120 volts. Further gels were stained with coomassie blue.
2.7 Western blotting

Using submerged transfer apparatus (Biorad Apparatus) at 150volts separated proteins were transferred to Hybond™-C Extra (GE Healthcare) membrane was blocked in 5% BSA over night at 4 °C. Membranes were incubated with P13 antibody (polyclonal antibody were raised towards CNBr digest of collagen type I) with a dilution of 50 µg/ml, washed with TBS 0.2 % Tween followed by HRP-linked secondary IgG (donkey anti-rabbit at 1:50,000, GE Healthcare) and developed with luminol (Sigma).

2.8 PCR genotyping

PCR was carried out using three primers (P1 common forward 5’CTTAGACACCTGCTACGGGC-3’, P2 neo reverse 5’-CACGAGACTAGTGAGACGTG-3’, P3 β reverse 5’-CCTGCCTGAGGCTGAGTG-3’) for genotyping of tail DNA from β3 integrin knock out mice. PCR reaction was carried out in 20 µl of PCR mix containing 19.47 µl of PCR buffer and 0.5 µL of tail DNA digest supernatant and PCR program was carried out at 95°C for 5min, 95°C for 30 s, 66°C for 30 s, and 72°C for 1.30 min and was run for 30 cycles followed by 72°C for 7 min. PCR products were analyzed by 1% agarose gel electrophoresis and Ethidium Bromide staining as shown in Figure 2 a, b and 3. The 446-bp band corresponds to the wild-type allele and the 538-bp band corresponds to the knockout allele.
Figure 1. Biosynthetic pathway of collagen synthesis and assembly process.
3. Results and Discussion

PCR analysis was performed using three primers (P1 common forward 5’CTTAGACACCTGCTACGGGC-3’, P2 neo reverse 5’CACGAGACTAGTGAGACGTG-3’, P3 β reverse 5’-CCTGCCTGAGGCTGAGTG-3’) for screening the β3 integrin knockout mice in each PCR tube. Homozygous knock out mice has no functional alleles of β3 integrin with both Neo alleles and showed only a 538-bp band the Neo band shows that animals are transgenic. Heterozygous mice has one functional allele of β3 integrin and the other Neo allele. Heterozygous mice showed both the 538-bp Neo band and a 446-bp band. Wild type mice has two normal alleles of β3 integrin and showed only a 446-bp band as shown in Figures 2 a, b and 3.

Figure (2.a) PCR genotyping analysis of β3 knockout mice staining of DNA using EtBr, lane M marker, lane C PCR control, 1. 9 ♀D +/+ wild type, 2. 9 ♀B +/- hemizygous,3. 9 ♀A -/- homozygous knockout, 4. 10 ♂A +/+ wild type, 5. 10 ♂D +/- wild type, 6. 11 ♂D +/- hemizygous,7. 11 ♀B +/- hemizygous.
(2.b) PCR genotyping analysis of β3 knockout mice. 9 ♀ A -/- homozygous knockout, 4. 10 ♂ A +/- wild type, 7. 11 ♀ B +/- hemizygous, 8. 12 ♀ A +/-, 9. 12 ♂ D +/- hemizygous, 10. 13 ♀ D +/- hemizygous, 11. 14 ♂ A +/- hemizygous, 12. 14 ♂ D +/- hemizygous, DC > Digestion control.
Figure 3. PCR genotyping analysis of β3 knockout mice staining of DNA using Gel Red Lane 1. F2 5 ♂ D Heteozygous +/−, Lane 2. F2 4 ♀ A Heteozygous +/−, Lane 3. F2 6 ♂ A Heteozygous +/−, Lane 4. F2 5 ♂ A, Homozygous +/+, Lane 5. F2 6 ♂ D Heteozygous +/−, Lane 6. F2 4 ♀ D Heteozygous +/−, Lane 7. F2 6 ♂ B Heteozygous +/−, Lane 8. F2 5 ♂ B Heteozygous +/−, Lane 9. F2 4 ♀ B Homozygous +/+, Lane C. PCR negative control, Lane Marker 100 bp.
Collagen extraction using acetic acid, pepsin, and sequential extraction with PBS, 0.5 M acetic acid followed by limited proteolysis with pepsin 3 mg/ml followed by salt precipitation of collagen type I with separation on SDS PAGE showed α1, α2 and β1, β2 chains were observed from the tumor samples as shown in Fig 4, 5 and 6. Collagen that was released directly by 0.5 M acetic acid from tumor samples as shown in Fig. 4 Collagen from tumor tissue that was not released by 0.5 M acetic acid was digested with pepsin. Collagen was released from pepsin treated tumor samples which were later salt precipitated and separated on SDS PAGE as shown in Fig. 5. Comparing the bands α1 and α2 collagen species in Gleevec treated mice and control (vehicle mice) did not show a major difference in collagen extracted from 0.5 M acetic acid fraction, Pepsin 3mg/ml fraction and sequential extraction with PBS, 0.5 M acetic acid followed by limited proteolysis with pepsin 3 mg/ml. These results indicate that Gleevec does not alter proportion of α1 and α2 ratio. We have also observed no difference in the amount of β bands.

Figure 4. SDS-PAGE on collagens extracted by direct 0.5M acetic acid fraction and further salt precipitated with 0.7 M NaCl and the tumor pellet from each fraction was dissolved in 50 μl of (2X) Sample buffer. 5 μl from the each samples were loaded on 6 % SDS-PAGE. Lane M- Marker, Lane 1 – Gleevec treated mice, Lane 2 – Vehicle treated control mice, Lane 3– Vehicle treated control mice, Lane 4– Vehicle treated control mice , Lane 5 – Gleevec treated mice , , Lane 6 – Gleevec treated mice.
Figure 5. SDS-PAGE collagen extracted by 0.5 M acetic acid containing 3 mg/ml pepsin, further 0.7 M NaCl salt precipitated and pellet from each fraction was dissolved in 50 μl of (2X) Sample buffer. 10 μl from the each samples were loaded on 6 % SDS-PAGE. Lane 1 – Glevec treated mice, Lane 2 – Vehicle treated control mice, Lane 3 – Vehicle treated control mice, Lane 4 – Vehicle treated control mice, Lane 5 – Glevec treated mice, Lane 6 – Glevec treated mice.
Figure 6. SDS-PAGE collagen extracted by 0.5M acetic acid containing 3mg/ml pepsin, further salt precipitated with 0.7 M NaCl salt and pellet from each fraction was dissolved in 50 μl of (2X) Sample buffer. 10 μl from the each samples were loaded on 6% SDS-PAGE. Lane 1 – Glevec treated mice, Lane 2 – Vehicle treated control mice, Lane 3 – Vehicle treated control mice, Lane 4 – Vehicle treated control mice, Lane 5 – Vehicle treated control mice, Lane 6 – Glevec treated mice.
To check the sensitivity and specificity of P13 antibody (a polyclonal antibody raised against CNBr digest of collagen type I) experiments as described in methods 2.2, 2.3 and 2.4 were performed. These experiments indicate that P13 antibody molecule detects both \( \alpha_2 \) and \( \beta_1;2 \) collagen species as shown in figure 7 and 8. Immunoprecipitation experiments on native collagen with different concentration of antibody and without antibody used to precipitate same amount of collagen did not show any major difference in the bands obtained in Western blot as shown in Figure 7. The reason could be due to lack of immunoprecipitation with P13 antibody and also may be due to direct binding of collagen with Sepharose. Experiments were performed on the supernatant fraction from collagen extracted from sequential method from CT26 tumors in order to check if there exits any free \( \alpha \) or \( \beta \) chains. Immunoprecipitation experiment from CT26 supernatant showed a \( \alpha_2 \) band in one of the control mice supernatant fraction but could not detect any other band in other control and Gleevec treated mice as shown in Fig 8.
Figure 7. Western blot of native collagen (vitrogen), Lane 1. Native collagen 1 µg, Lane 2. Native collagen 3.1 µg, Lane 3. Native collagen 12.5 µg, Lane 4. Native collagen 37.5 µg concentration was made up to 12.5 µl with PBS and 12.5 µl of (2X) Sample buffer to make up 25 µl to each of the samples from lane 1-4, Lane 5. Collagen at a concentration of 200 µg/ml prepared by dissolving in 1x PBS immunoprecipitated with 75 µg of 1* Ab., Lane 6. Collagen at a concentration of 200 µg/ml prepared by dissolving in 1x PBS immunoprecipitated with 100 µg of 1* Ab, Lane 7. Collagen at a concentration of 200 µg/ml prepared by dissolving in 1x PBS immunoprecipitated with 150 µg of 1* Ab, 200 µg of collagen. Lane B. collagen at a concentration of 200 µg/ml prepared by dissolving in 1x PBS without 1* Ab, Lane M. Marker.

Figure 8. Western blot of denatured collagen and CT 26 tumor samples from acetic acid and pepsin supernatant 1 a/b fraction. Lane 1. pH 7 collagen 200 µg/ml heat denatured 55 °C for 20 min. Lane 2. pH collagen 200 µg heat denatured 55 °C for 20 min at higher pH. Lane 3-8 Supernatant fraction from 1 a/b sequential fraction after collagen extracted pH was adjusted to 7 using NaOH solution immunoprecipitation was performed by adding 100 µg of P13 antibody and 100 µl of Sepharose was added. Lane 3. Vehicle. Lane 4. Vehicle. Lane 5. Vehicle. Lane 6. Gleevec treated, Lane 7. Gleevec treated, Lane 8. Gleevec treated. Lane 9. Marker.
To check whether CT26 tumors synthesis of collagen in vitro. CT26 cells were cultured in T-flask until cells reach confluency. Collagen from the cultured cells was extracted using acetic acid pepsin and salt precipitation. Collagen extracted from the CT26 cells cultured in vitro showed a α2 band in western blot as shown in Fig 9. However mostly collagen synthesis in tumors is considered as host response towards the tumors. Supernatant fraction from Immunoprecipitation experiment from CT26 tumors showed no band in western blot as shown in Fig 9. This experiment was performed to check if there is any collagen present in supernatant fraction after Immunoprecipitation.

Figure 9. Western blot of collagen extracted from CT26 culture invtro 0.5M acetic acid and 6mg/ml pepsin further precipitated with 0.7M NaCl and supernatant fraction after pH adjusted to 7 ;C- CT26 Cell fraction isolated collagen, V- Vehicle supernatant after pH 7 , G- Gleevec treated supernatant after pH 7 , M-Marker.
Ex-vivo system of collagen synthesis was performed to investigate the role of αvβ3 integrin role in collagen synthesis in tumor tissue. Collagen extracted using solubility in pepsin acetic acid fraction and salt precipitation from tumor tissue explants supplemented with medium containing radioactive glycine and proline as described in method 2.1 and separated on SDS PAGE as shown in Fig.10. Collagen turn over studies shows that radiolabelling of collagen with glycine and proline was successful only in two samples (integrin β3 knockout 1001D and integrin β3 wild type). Comparison made between these two samples indicate that collagen turn over was higher in integrin β3 knockout 1001 than wild type, as band intensity of α1 and α2 was high in integrin β3 knockout as shown in Fig11. However absence of radiolabelling of collagen in different tumor samples was observed which may be due the time gap taken for the exvivo cultures which may lead to death of malignant cells. The other reason could be due to heterogeneity within the tumor samples and third reason may be due necrotic tissue in may be present in tumor explant used for Ex-vivo study.

Figure 10, coomassie staining of collagen extracted by 0.5M acetic acid and 6mg/ml pepsin further precipitated with 0.7M NaCl from Itgb3 knockout and wide type tumors. M-Marker lane, Integrin β 3 Knockout mice 1001A, 1001B, 1001D; Integrin β3 wild mice 1005D, 1006A, 1006B
Conclusion

This study shows evidence through Ex-vivo system model for collagen turnover. Collagen turnover is altered in two out of six samples so this study remains inconclusive. However further work needs to be done to validate this study by increasing the radiolabelling in tumor tissue. This study shows that treatment of CT 26 tumors with Gleevec didn’t showed any alteration in collagen synthesis.

Future projects

Our study suggests that $\alpha_\beta_3$ integrin plays a possible role in collagen turnover. This may suggest that, as cancer cell growth and metastasis involves collagen remodeling, so there exists a possible role of $\alpha_\beta_3$ integrin in cancer microenvironment during the process of growth and metastasis.
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