The Characterization of Tumor Microenvironment in Tumors With and Without the TGF-β Signaling

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BRIEF: The goal of this work was to characterize TGF-β signaling in the tumor microenvironment.

ABSTRACT: Transforming growth factor beta (TGF-β) has been identified as a major component of tumor initiation and progression. However, the mechanism of TGF-β and its role in epithelial cells and adjacent stroma tissue remains unknown. To address the role of TGF-β, two experiments were developed. The first model implanted fibroblast tissue and tumor cells in samples with or without TβRII (a protein receptor express TGF-β signaling), into chicken embryos. The second model implanted tumors treated with BAPN, an inhibitor for lysyl oxidase (LOX), into lacking TGF-β signaling mice. To identify the changes in cell migration and tissue formation, immunohistochemistry experiments were used. The results indicate the control tumor cells with fibroblasts enhanced collagen expression, and a greater presence of fibroblast-like cells. The tumors lacking TGF-β signaling with BAPN restriction on LOX have a more aggressive tumor microenvironment, more organized collagen network, and reduced greater amount of LC3B, a protein involved in autophagy. Overall, the tumors lacking TGF-β signaling have a more aggressive tumor microenvironment than the control tumors.

INTRODUCTION.

Cancer is one of the major causes of human fatality. Statistics from the American Cancer Society indicated that there will be 1.5 million new cancer diagnoses and 562,340 people are expected to die from cancer in 2009 [1]. Cancer cells are not similar to other trillions of normal cells. They have the ability to grow and divide constantly, and form different phenotype. The ability to metastasize, or spread from an organ to other parts, makes cancer known as a very deadly disease.

Transforming Growth Factor Beta (TGF-β) is one of the major cell initiators. It plays an important role in human cancer cells. Studies have shown that TGF-β signaling was found to promote tumors in breast, colon, esophageal, gastric, lung, pancreatic and prostate cancer [2]. It has a special dual role, functioning as both a tumor suppressor (limit the growth of tumor cell) in the early stage and tumor promoter (stimulate the growth of tumor cell) in the later stage [3]. Research has shown that cells without TGF-β signaling showed early tumor latency (first day of tumor detection) and increased lung metastases [4].

With multiple functions, TGF-β affects the interactions with the adjacent microenvironment (cells, vessels, and other molecules that surround a tumor). A presence of TGF-β signaling in tumors, the epithelial cells will form a single-cell migration [5]. Without the presence of TGF-β, epithelial cells will form the collective-cell migration (many cell stick and migrate together), and increase the expansion of stromal cells [4].

TGF-β is not the only cell initiator that can increase tumor metastasis. Other metastasis-associated genes, such as, Lysyl oxidase (LOX) may also have the potential to increase metastasis. LOX is a copper dependent amine oxidase locates in stromal region. It has the potential to cross link collagens fibers or elastin in tissue. LOX can increase the strength and structural integrity between linkages of fiber tissue to itself [6].

The mechanism of TGF-β signaling and its role in epithelial cells and adjacent fibrovascular stromal interactions represent a great interest of the scientific community. Therefore, it is important to understand the characteristics of the microenvironment in tumors with and without TGF-β signaling. The study hypothesizes that tumors lacking TGF-β signaling will have a more aggressive tumor microenvironment than tumors with TGF-β signaling. To address the question, two models were developed. One model implanted fibroblast tissue and tumor cells in samples with or without TβRII (a protein receptor expressed in TGF-β signaling), into chicken embryos. The other model implanted tumors treated with BAPN, an inhibitor for lysyl oxidase (LOX), into lacking TGF-β signaling mice. By using these designs, the experiment will be able to show the difference in the microenvironments in both tumors with and without TGF-β signaling.

MATERIALS AND METHODS.

Tissue procurement.

The chick embryos, *Gallus gallus domestics,* were injected with both mouse tumor cells with TGF-β signaling with fibroblast, and mouse tumor cells without TGF-β signaling with fibroblast. The injection was inserted onto the Chorioallantoic membrane (CAM), the most outer membrane of chick embryos. The injection was inserted on day 10, with total of 3 million cells/25μl (2.5 fibroblasts: 1 epithelial cell) in each egg [7]. The chicks were sacrificed on day 19 or day 20, right before the hatch day, on day 21.

Mouse model.

The mouse, *Mus musculus,* had been genotyped with MMTV-PyMT/MMTV-Cre/TβRII $^{fl/fl}$), a cell line that does not have TGF-β signaling due to the removal of TβRII receptor [8]. The BAPN water treatment began on the development day 28 [9]. The BAPN treatment will disconnect the bonding of lysyl oxidase (LOX) with fiber tissue. The mice were sacrificed 28 days after BAPN treatment.

Immunohistochemistry and immunofluorescence.

Immunohistochemistry and immunofluorescence were all conducted by using standard protocols. The staining for collagen on both chick and mouse tissues, was conducted by staining tissues with the Picrosirius Red, along with washes of acidified water, 100% ethanol, and xylene. Immunohistochemistry was also used to indicate Lyve-1(anti-Rabbit) expression with the mouse tissue. The tissue was blocked with 500μl of goat antibody and 4.5 ml of PBS with 1:400 dilutions of primary goat and 1:1000 dilutions of secondary goat antibody. The immunofluorescence for the chick tissue was conducted by using the antibodies cytokeratin 8/18, α smooth muscle actin, and Dapi. Cytokeratin 8/18 expresses the epithelial cells in the tumors by using guinea pig primary antibody at the dilution of 1:500, and goat anti-guinea pig 594 secondary antibody(Invitrogen AlexaFluor) at the dilution of 1;1000. The α smooth muscle actin (αSMA) expresses the collective cell migration or fibroblasts-like cells located in the stromal by using mouse primary antibody at the dilution of 1:50 and donkey anti-mouse 488 secondary antibody (Invitrogen AlexaFluor) at the dilution of 1:1000. Dapi was used to express the location of the nucleus.

Western Analyses.

After proteins were extracted from mouse blood, the western was prepared by using 30ml of running gel with 4% concentration of master mix solution and 5% concentration of stacking gel. After the proteins were transplanted onto the membranes, the primary antibodies E-cadherin, Vimentin, Profilin-1, PARP, and LC3B were applied. In the secondary antibodies, the sample concentrations were mouse at the dilution of 1:2500, chicken at the dilution of 1:2000, and rabbit at the dilution of 1:1000. All the solutions of antibodies were at the dilution of 1:500 with 5% milk.

RESULTS.

Mouse tumors without TGF-β signaling in chick embryo increase collective epithelial cell formation and decrease fiber-like cell migration.

To test the effect of TGF-β signaling on the microenvironment in the chick embryo, immunofluorescence was conducted. The antibodies cytokeratin 8/18 amplify the epithelial cells, and α Smooth Muscle Actin amplifies the fibroblast-like cells in the stromal area to visualize the differences in cell migration and number of cells located at the stroma (Figure 1). The observation of tumors with TGF-β and tumors without TGF-β has a different epithelial cell formation. With the presence of TGF-β and fibroblast tissue (control), the epithelial cells are more scattered, individual, and exhibit a more singular formation (Figure 1a). However, tumors without TGF-β and fibroblast tissues (KO), the epithelial cells are group as a whole and exhibit collective formation (Figure 1e). As for the difference in stromal cell region, control with fibroblast tissues had a higher presence of fibro-like cells, which can indicate more singular cell migration (Figure 1b). On the other hand, the KO and fibroblast had less presence of fibro-like cells, which has less singular migration. As shown in Figure 1d and 1h, there is more heterogeneity of epithelial and fibro-like cells in the control tumors than in the knockout tumors.
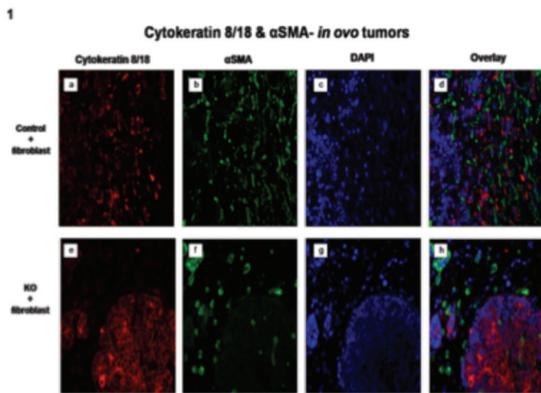

Figure 1. Immuno-fluorescence of tumor sections. Cytokeratin 8/18 express epithelial cells in red (**a,e**). α Smooth Muscle actin express fibro-like cells or migration cells in green (**b,f**). Dapi staining indicated the nucleus of cells in blue (**c,g**). The overlay of all cells (**d,h**). Epithelial cells in the control + fibroblast tumors exhibit mesenchymal characteristic (αSMA) in stromal regions, while the KO + fibroblast tumors show collective formation of the epithelia with limited expression of αSMA in the surrounding stroma.

Mouse tumors without TGF-β signaling implanted in chick tissues have a lesser concentration of collagen expression.

Histological analysis was used to test the effect of TGF-β signaling on collagen fibers. In the absence of TGF-β and fibroblast tissues, there is a lower concentration of collagen fibers and most of collagen fibers surround the epithelial cells expressed in the stromal area (Figure 2b,d) In the presence of TGF-β and fibroblast, there is a higher concentration of collagen fibers, and the collagen fibers are mostly expressed within the epithelial cells (Fig. 2a,c).
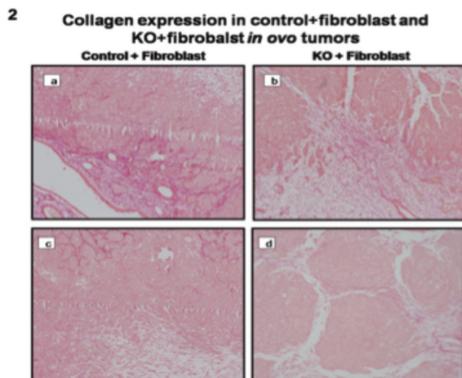

Figure 2. Picrosirius Red Staining of tumor sections. Darker pink color equates to higher collagen expression. Collagen fiber expression is greater and more dispersed in control + fibroblast tumors. However, the collagen present in the KO + fibroblast tumors is more organized around individual tumor nodes.

Mouse tissues without TGF-β signaling and with BAPN treatment have less organized collagen fibers.

The mouse tissues without TGF-β signaling were treated with BAPN. BAPN treatment was able to channel out the bonding of lysyl oxidase (LOX) with collagen fibers. By using histologic analysis, it indicated that the tumor without TGF-β signaling with BAPN treatment has less organized collagen fibers in the stroma (Figure S1b, d), than the tumor without TGF-β signaling with no BAPN treatment (Figure S1a, c).

Mouse tissues with the absence of TGF-β signaling and without BAPN treatment have more LC3B protein expression.

By testing the mechanisms of tumors lacking TGF-β with and without BAPN treatment for multiple protein expression, there were no significant differences in protein E-cadherin, Vimentin, Profilin-1, and PARP (Figure 3). However, the only difference in protein expression was LC3B, which had a higher expression in tumors without BAPN treatment. The experiment indicated that there was more expression of cell death in tumors without BAPN treatment.
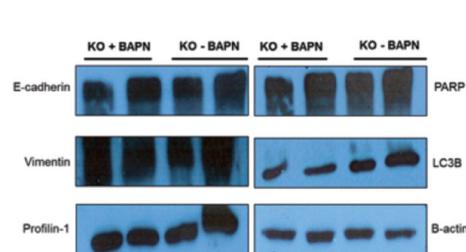

Figure 3. Protein expressions of E-cadherin (ce*Lacking* adhesion), Vimentin (fibroblasts- like cell), Profilin1 (cellular migration), and PARP (cell death) did not show a significant difference in KO with BAPN and without BAPN. However, there was a difference in protein expression LC3B (cell death) which KO without BAPN treatment has a greater LC3B expression than KO with BAPN.

Mouse tissues without TGF-β signaling and BAPN treatment have less expression of LYVE-1 + cells.

By observing the difference of lymphatic concentration in mouse tissues, the experiment indicated that KO with BAPN treatment have less concentration of LYVE-1+ cells (SF. 2b,d), and KO without BAPN treatment have a greater LYVE-1 + cells expression in the tumor (SF. 2a.c).

DISCUSSION.

TGF-β regulation on cellular migration.

The hypothesis of this project was that tumors lacking TGF-β signaling will have a more aggressive tumor microenvironment than tumors with TGF-β. TGF-β plays an important role in regulating cellular differentiation and migration [4]. The data support the hypothesis that without TGF-β signaling, the epithelial cells will collectively migrate, which promotes massive tumor metastases.

Without TGF-β signaling, tumors can also increase carcinoma associated fibroblasts, which contributes to cellular migration and progression that induces metastases [2]. However, the result of chick embryo model showed that there were more fibroblast-like cells in the control fibroblast tumors than in KO fibroblast tumors (Fig 1). Although there was a higher expression of fibroblast-like cells in the control, it is not known specifically whether those cells are a singular formation of epithelial cells or adjacent fibroblast. When the epithelial cells move closely out of the cell membrane, the characteristic of an epithelial cell will tend to have a more longitudinal shape which is similar to fibroblast tissues [10].

TGF-β regulates different collagen fibers development.

It is well known that the loss of TGF-β signaling can have an effect on inducing adjacent microvascular cells [4]. The data showed that in the control chick tumors with fibroblast, the tumors have a more expression of collagen fibers

and a more dispersed tissue in stroma. However, the collagen fibers in the KO with fibroblast tumors are more organized around the individual tumor nodes.

TGF-β with BAPN treatment regulation on collagen fiber.

LOX is an enzyme that forms a strong bond between fibers, which is found in the fibroblast located in the stroma. Some studies have shown that LOX increases fibroblast cells and induce metastases [6]. In the results, the sample without TGF-β signaling and BAPN treatment tumors cause the collagen fibers to develop differently in the stroma.

There is more collagen fibers expression in tumors lacking TGF-β with no BAPN treatment. The collagens were organized around the epithelial cells.

LC3B protein expression is correlated with increased cell death in the KO – BAPN.

Several studies have determined that LOX is essential for hypoxia related cell death [11]. In the Western blot for protein LC3B (Figure 3), it illustrated that KO without BAPN treatment has a higher expression of LC3B.

Loss of TGF-β signaling and BAPN treatment regulates LYVE-1 expression.

One of the major microenvironment components is LYVE-1 expression, which occurs in the lymphatic vessels. The results determined that the treatment with BAPN in KO tumors significantly reduces the number of lymphatic vessels and LYVE-1 infiltration myeloid cells.

Future studies will focus on determining whether migration of cells in the chick embryo are fibroblast-like cells or epithelial cells, both of which are located in the stromal areas. It will also be important to understand the relationship between LOX and hypoxia and alterations in cell death for the KO tumors that have been treated with BAPN.

SUPPORTING INFORMATION.
Figure S1. Picrosirius Red Staining of TRBII KO mouse tumor sections
Figure S2. LYVE-1 immunohistochemistry conducted on TβRII KO tumor sections.

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