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Cancer Associated Fibroblasts: Creating a Suitable Environment for Cancer

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Clinical significance: Myofibroblasts-a cozy bed for cancer cells?

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ABSTRACT:

Cancer associated fibroblasts (CAF) may be major players in cancer together with the malignant epithelial cells. However, the origin of this CAF remains an enigma.

Objective: Is CAF a normal fibroblast that has transformed itself under the influence of the growth factors or is it the effect of the malignant epithelial cells? Alternatively, has it formed from cancerous epithelial cells, or endothelial cell by EMT (epithelial mesenchymal transition)? On the other hand, has it originated from the stem cells, the mesenchymal bone derived cancer stem cells?

Materials and Methods: Published articles were identified through a literature search using online databases such as Scopus, Medline, and Google, for papers published from the year 2004 to 2016 using the key words; oral carcinogenesis, EMT, cancer associated fibroblasts/ myofibroblasts, endothelial cells,

for articles written in English. For inclusion, publications had to meet pre-determined criteria. The author selected such articles from journals, clinical reports, and web links.

Results: The origin of cancer associated fibroblasts (CAF) in cancer, though extensively studied, remains unanswered.

Conclusion: Knowledge of the source of the myofibroblasts that create a favorable environment for the progress of the cancer is beneficial in understanding cancers, including oral cancer. Drugs administered with the aim of targeting this microenvironment to eliminate the cancer associated fibroblasts would result in complete remission of the cancer.

Keywords: cancer; epithelial mesenchymal transition (EMT)**;** endothelial cells; TGF-β; MSCs (Mesenchymal bone- derived stem cells).

INTRODUCTION

Epithelium and connective tissue are strikingly different in- terms- of its origin, structure and function. They juxtapose each other even as they are separated by a thin double- layered basement membrane, which is semipermeable, and restricts the easy entry of cells, water, organisms, proteins and other biochemical materials. Epithelial cells are known to play a major role in cancer, but it is only in the recent years, that the role of the connective tissue stroma was considered equally important. From the histological perspective, cancer was considered “a wound that never healed." All the cells that are engaged in wound healing, like the fibroblasts, endothelial cells and macrophages, etc. play a role in the pathogenesis of cancer.[1]

Located between the cancer cell and the carcinoma in-situ cell and the surrounding tissue of fibroblasts and extracellular matrix is a basement membrane, which is made up of a thin double- layered sheet of fibers and proteins that normally cradles the cells above it. The basement membrane is also the leading physical barrier that keeps primary epithelial tumor cells from seeping into the matrix below. Perforating the basement membrane is a cancer cell's first move towards invasion. Fibroblasts and the altered fibroblasts, also called as the cancer associated fibroblasts or myofibroblasts, are the most predominant components in the connective tissue, and they synthesize the new extracellular matrix and fibers, to create a suitable environment for cancer. These altered cells may play a role in making the basement membrane permeable to the overlying cancer cells.[1]

DISCUSSION

Connective tissue, which is primarily composed of collagen fibers, is said to secrete a large number of cytokines/growth factors and enzymes such as collagenases. Growth factors may be autocrine or paracrine in nature. TGF-β is an important growth factor, which induces the formation of fibroblasts to help in breaching of a wound. The fibroblasts that help in wound closure are unique and different from the normal fibroblasts and are called as the 'myofibroblasts'. The myofibroblasts have dual properties, that of a smooth muscle and of a fibroblast. In cancer, these myofibroblasts are a part of the cancer associated fibroblasts (CAF), and are thought to participate actively in the growth and invasion of the tumor cells into the underlying connective tissue. It provides an exclusive tumor'microenvironment', which consists of a vibrant mixture of fibroblasts, endothelial cells, monocytes/macrophages, and granulocytes.[1]

Recent studies have shown the existence of the myofibroblasts in oral malignancies and potential malignancies. It has been identified immunohistochemically in oral submucus fibrosis, oral dysplasias and oral carcinomas.[2]

Myofibroblasts, in normal and cancer cells, have contractile properties. They have a tendency to adhere tightly to each other by fibronexus making the collection of the myofibroblasts dense. An adhesive protein, fibronectin, facilitates Fibronexus. A dense collection of collagen fibers or myofibroblasts could be a breeding ground for the cancer cells.Thisfacilitates the invasion of the cancer cells into the connective tissue with the help of enzymes such as the MMPs (matrix metalloproteins) and the catalases. This hypothesis arises from the experimental evidence, of epithelial cells are exposed to MMP-driven oxidative stress undergo DNA oxidation and experience mutations, thereby undergoing EMT in which they transdifferentiate into activated myofibroblasts.[3]

Histologically, the fibroblasts are identified by their fusiform architecture. Myofibroblasts, on the other hand, are more plump and large as opposed to normal fibroblasts, which have thin and wavy nuclei.[4] Markers for myofibroblasts have been shown to be positive for α-SMA and hence found similar to a muscle tissue. Other positive markers in myoepithelial cells, apart from the smooth muscle actin (SMA), are the fibroblast stimulating protein-1 (FSP-1), platelet-derived growth factor α (PDGFR-α), and PDGFR-β [5] and other less accepted markers such as the transcription factor S100A4.[5]

Since there are other cells and tissues as the vascular smooth muscle (SM) cells that are α-SMA positive, it cannot be used as a marker for the myofibroblast. However, unlike the myofibroblasts, the SM cells express α-SMA and other markers, in the later stages of its formation. Other markers that are introduced late in the life of the SM cells are for e.g.desmin, SM myosin smoothelin and h-caldesmon,and these are not expressed by the myofibroblasts. Pericytes are also positive for α-SMA and have other markers common to myofibroblasts, but they lack the 'stress fibers' seen in myofibroblasts. Since the myofibroblasts have multiple sources of origin, the term "cancer associated fibroblasts" seems favorable for these altered fibroblasts. [6]

There is a consensus regarding the composition of the cancer cells; that is, it is composed of modified normal cells that have been acted upon by certain agents. Its stroma is made up of structures and cells such as the myofibroblasts, bone- derived mesenchymal cells, leucocytes etc. The stroma is found to play a dual role: on the one hand, in protecting the body against cancer and on the other hand in helping in the promotion of tumor growth.

The role of the stroma microenvironment, especially the fibroblasts; in the initiation, enlargement and the spread of the neoplastic cells was found to be important. This theory was proposed based on Paget's "Seed and Soil" hypothesis of 1889, on growth and metastasis of cancer. The fibroblasts have been found to escort the cancer cells through the various steps in carcinogenesis. Signal proteins have been discovered transmitting messages to and from the epithelium and stroma. The focus is now on the molecular and cellular features of the specialized fibroblasts so that it may help in the prevention, prognosis, and treatment of cancer.

Recent articles have focused on the genetic and epigenetic changes in the fibroblasts that have altered the genes, that help in the formation of the growth factors and cytokines, which act on normal fibroblasts. The altered microenvironment if considered a soil,' the altered growth factors increases its ‘fertility’ to enhance the growth of the ‘seed’ i.e. the tumor.

For the tumor formation to proceed and spread, the epithelial mesenchymal interaction is required. To progress and spread, tumor cells need to keep interacting with their stromal fibroblasts through mediators, again controlled by various other agents. The interaction is found to be reciprocal. Nevertheless, the salient question here is it the epithelium that is dysfunctional or is it the stroma? The authors have concluded that whichever be the tissue that initiates tumor formation, the main objective of both is to grow and cause expansion of the cancer cells.[7]

Myofibroblasts express characteristics common to both the smooth muscle cells and fibroblasts and are the main cell population found in CAFs. Stromal fibroblasts exposed to the medium that has been conditioned by carcinoma cells, differentiate into myofibroblasts. Under the directive of the growth factors, predominantly the TGF-β, they are more competent in promoting tumor growth. It is likely that carcinoma cells not only initiate the conversion of stromal fibroblasts into myofibroblasts but also help to maintain their activated phenotype *in vivo*. The presence of myofibroblastic CAFs within the cancer can corroborate the evolution of the normal stroma towards a tumor-promoting microenvironment.

"Tumor is a wound that never heals." All features playing a role in wound healing, also promote tumor formation. Myofibroblasts help in wound contraction and therefore helps in wound healing, and they are the important components of the CAF. Understanding the molecular crosstalk, which occurs between CAFs' and cancer cells is essential, and may in the future provide novel therapeutic targets for the treatment of cancer.

CAFs secrete various growth factors and cytokines into adjacent cancer cells such as TGF-β and hepatocyte growth factor (HGF). In the case of the breast cancer the increased expression of these two growth factors in mouse fibroblasts can encourage the initiation of cancer by co-introduction of normal epithelium. Experimental studies have shown the myofibroblasts to have arisen from different cell types such as the pre-existing fibroblasts, preadipocytes, smooth muscle cells, endothelial cells, epithelial cells and bone marrow-derived progenitors. Bone marrow-derived stem cells are a significant cellular source of myofibroblasts found in the tumorstroma. This is associated with an increased risk of invasion and metastasis and a poor clinical prognosis

Epithelial cell conversion into myofibroblasts by EMT is known, but not much detail is available about the stroma's role in inducing proliferation of cancer epithelium. Tumor associated myofibroblasts evolves, together with the neoplasia; from malignancy to a more severe malignancy and this interaction may be targeted, to lead to the development of the novel therapeutic approaches to treating cancer.[8]

Epithelial tissues such as the skin, liver, lung, heart and kidney react to injury and disturbance in tissue homeostasis by production of the myofibroblasts. Myofibroblasts are also said to form by EMT from local epithelial and endothelial precursors during tumor development, as well as in the lung, kidney fibrosis, heart and liver fibrosis. Added to that, the SM cells may also undergo de-differentiation into ECM synthesizing cells to form more myofibroblasts, which further contributes to the myofibroblast population.

The tumorstromainsystemic sclerosis, vessel repair, dermal scarring and the pericytes has been stated to acquire contractile myofibroblasts properties.

Other sources for the CAF includes the bone marrow derived MSCs and blood circulating fibrocytes. These are recruited into the inflamed and remodeled tissue. But the fibrocyte to the myofibroblast differentiation cannot be considered as the rule of the thumb.

Probable source of origin of the myofibroblasts in CAF include:

1. Epithelial, endothelial cells, adipocytes and pericytestransform into mesenchyme through Epithelial Mesenchymal Transition (EMT).
2. Mesenchymal cells or mesenchymal bone derived stem cells (MSCs) transforming into CAF.
3. Fibroblasts, fibrocytes by incorporating the α- SMA transforms into CAF.[7]

Epithelial cells undergo a transition to CAF through EMT. The epithelial mesenchymal transition is a normal occurrence during embryogenesis for eg. The embryological neural crest cell, which is ectodermal in nature, descends into the various parts of the body to initiate development of various organs such as

the jaws; the teeth, ears, nose, etc. and acquire ectomesenchymal characteristics i.e. both ectodermal and mesenchymal characteristics.

Epithelial-mesenchymal transition (EMT) describes the transformation of the epithelial cell with tight junctions to a mesenchymal cell with loose junctions. A special type of the EMT is seen in malignancy where there is the transdifferentiation of the myofibroblasts from epithelial cells, instead of transforming into malignant epithelial cells. In case of breast carcinoma the epithelial cells undergoes transdifferentiation into myoepithelialcells, which further differentiate into myofibroblasts, the ancestors of the CAF. The mouse, skin carcinoma cells lose its cell adhesion marker namely the E-cadherin and transform itself into a myofibroblast through the Ras and TGF- β signaling. The CAFs formed may not be as malignant as the epithelial cells in most cases, but it would encourage and promote the growth and the metastasis of cancer. In case of cancer of the kidney it has been shown that its CAF contains a large content of β- galactosidase which is predominantly an enzyme of its' epithelial cell. Similarly in the breast cancer CAF, genetic markers, indicates its derivation from the epithelial cells. The CAF is also said to be derived from the normal surrounding normal epithelial cells as an additional source since the entire CAF could not have been derived from the cancer cells.

CAF from adipocytes

Since the origin of the CAF is under speculation, studies have been conducted to study the role of adipocyte stem cell as a source of CAF. Since adipocytes have been found in close association with the cancer cells of the breast, this probability is being looked into. Experimentally these cells under the influence of the TGF-β have been found to convert into the CAF. TGFβ1 signaling occurs via Smad3 conversion of hASCs (human adipocyte stem cells) into the CAF. These hASC-derived CAF-like cells showed all the functional properties of the CAFs, including that of tumor cell invasion and increased expression of stromal-cell-derived factor 1 (SDF-1) and CCL5. Hence it could be considered from this data that hASCs are a source of CAFs which play an important role in the tumor invasion. [9]

Origin of the CAF from endothelial cells through the EndMT

The TGF-beta was able to induce the proliferating endothelial cells to undergo a phenotypic EMT or the EndMT conversion into the CAF in cancer. The CAF cells then contained additional markers such as the fibroblast specific protein-1(FSP1) and reduced CD31/PECAM. Endothelial cells exhibited different phenotypes according to the local microenvironment. This has been observed in the melanoma and the pancreatic cancer models. The mural cells or the pericytes surrounding the blood vessels also shared a similar phenotype and markers with myofibroblasts, suggesting another likely source of derivation from the vascular tissue. Cancer cells require nutrition, which is provided by the blood vessels. The formation of the additional blood vessels are said to be under the control of an angiogenic switch that controls both angiogenesis and vasculogenesis(cancer blood vessels and normal blood vessel respectively) The

increased blood vessel count takes place under the direction of the angiogenic and antiangiogenic growth factors like the VEGF and the thrompospondin-1(TSP-1) respectively, released by the CAF. But the blood vessels formed by this mechanism in the cancer tissue, are fragile and leaky. The VEGF activates blood vessels through the VEGF receptor kinases. TSP-1 acts on receptors on the blood vessels and suppresses its formation. Both these factors play a predominant role in cancer formation and progression, and in the role of the CAF.[10]

Role of TGF- β in induction of CAF in epithelial cancers

As the tumor formation proceeds, it becomes unresponsive to the tumor inhibitory action of TGF-β1, and together with its angiogenicfavoring effect and its capacity to encourage EMT of cancer cells, assigns TGFβ1 a primary role in tumor development. The cell- to- cell junctions of the vascular endothelial cells are disrupted, due to the action of the TGF-β1, thereby facilitating the leakage of the metastasizing tumor cells. If the metastasis needs to be blocked, the action of the TGFβ1needs to be blocked because the TGFβ1 causes increased fibrosis and mediates the inflammation, resulting in the increased ECM production and reducing the synthesis of matrix metalloproteinases (MMPs), and hiking the production of tissue inhibitors of MMPs (TIMPs). It also enhances the retention of the hyaluronan, a glycoprotein in the ECM and also promotes the myofibroblast activation and survival, which completely feeds back on myofibroblast activation. So it is the action of TGF-β1 on the ECM producing the myofibroblasts and further secreting a larger quantity of TGF-β1 and hence continuing the cycle. This is the autocrine feed-forward loop;that is an attribute of the persisting myofibroblast activities, identical to that observed in fibrosis. [6]

The actin cytoskeleton during the EMT is reorganized to transform into actin stress fibres from its cortical adherens- associated location. This contributes to the formation of filopodia thereby encouraging cell migration. The process is also responsible for cancer invasion and spread. This is done by down regulating the E-cadherin an epithelial intercellular protein, thus releasing β-catenin to which it was attached, thereby increasing the expression of c-MYC, cyclin D1, and MMP7. This promotes the invasiveness of the cell. Together withthis, there is an increased secretion of the extracellular proteases and decreased ECM proteins, that further plays a role in cancer cell invasion. Mesenchymal markers and suppression of epithelial genes control the key transcription factors, therefore, controlling the EMT. The markers include the Twist, zinc-finger proteins Snail and Slug, proteins such as the ZEB-1 and -2, together with factor FoxC3, which are all under the regulation and control of TGF-β. In the skin, melanocytes are tightly adhering to the keratinocytes through the E-cadherin whereas in melanoma, there is an alteration in this communication under the influence of the TGF-β resulting in the EMT and allowing the melanoma cells to reach the connective tissue to adhere and communicate with fibroblasts and endothelial cells, hence encouraging their spread through the stroma. In osteosarcomas, on the other hand, the TGF-β downregulates the integrin α3β1 and reduces its adhesion to the substrate laminin.

However, the substrates such as collagen (α2β1 integrin) and fibronectin (α5β1 integrin) are not affected by TGF-β.

Cells migrate by generating cytoplasmic extensions called as lamellipodia and simultaneously retracting the trailing end. These actions are under the control of the Rho-family GTPases which are further under the control of the TGF-β. Other proteins that the TGF-β secretes to help in migration includes the metalloproteinases and the liberation of plasmin which, further plays a role in the release of stored TGF-β from the ECM, thereby increasing cell invasion.

Mesenchymal cells as a source of CAF

The mesenchymal bone derived stem cells is said to play a role in CAF formation. These stem cells play a role in extracellular matrix formation in normal circumstances. In the case of cancer, the CAF associated with it is found to show the immunohistochemical markers of the bone derived mesenchymal cells. The CAF is positive for α-SMA and vimentin and negative for myosin, desmin, and cytokeratin. Cancer progression results in the increase of the α-SMA and myofibroblasts (MFs) from the niche cells normally present in the bone marrow. The CAF derived from this source by the action of cytokines such as the TGF-β and SDF-1α, express IL-6, Wnt 5 and BMP 4 which shows the DNA hypomethylation and promotes the tumor growth in the case of the stomach cancer cells. Hence, the niche cells of the bone marrow shift to a cancer niche area when there is a tumor formation.[11]

Mesenchymal bone derived stem cells (MSCs), the multipotent stem cells, like all stem cells, have got the ability to self- renew and differentiate into a large number of mesodermal germ layers under ideal experimental in-vivo and in-vitro conditions. This results in the formation of cells such as chondroblasts, osteoblasts, and adipocytes. The MSCs undergo cell differentiation, wound healing and tissue regeneration in the presence of the growth factors like the VEGF (vascular endothelial growth factor), TGF (transforming growth factor, FGF (fibroblast growth factor), PDGF (plasma derived growth factor and IL-8 (interleukin -8), that helps in the migration of the MSCs from the bone marrow. The ‘tumor’ being similar to a ‘wound’ also causes the release of the MSCs that may play a role in the enhancement of the tumor and metastasis. The MSCs are also said to be ideal candidates, to be used as a drug delivery vehicle. Prolonged exposure to the tumor medium makes the MSCs transform into CAFs to become a part of the tumor microenvironment. The MSCs also promotes tumor growth by angiogenesis. A more improved perceptive of the core mechanism of the interaction between tumor cells and MSCs could result in the introduction of the novel treatment methods.[12]

CAF as the reason for tumor indurations

The extracellular matrix under the influence of the CAF undergoes tautness and this result in it getting stretched by the environmental forces, further resulting in a greater intracellular stress. Stiff ECM helps in

further conversion of the parent cell into myofibroblasts to a large extent and thereby keeps the latent TGF-β1, active. The CAF is stiffer than the surrounding stroma, and this clinical feature would be useful in the detection of the tumors by palpation or elastography. A high interstitial fluid pressure and increased lymphatic flow may be the other causes for “pre-stiffening”, within the tumor microenvironment. It has been experimentally proven that very low interstitial fluid flow is sufficient to initiate myofibroblasts formation. There is also a simultaneous occurrence of increased levels of the TGF-β1in the stroma, thereby further boosting the myofibroblastic transformation. [6]

Role of CAF in cancer

1. TGF-beta is the only known growth factor that can transdifferentiate fibroblasts into CAFs
2. The CAF also helps in the growth of the cancer cells by altering its environment.
3. Proliferating cancer cells prefer an anaerobic environment during carcinogenesis. They prefer an anaerobic glycolysis even when there is sufficient oxygen. CAF acts as a mutagen and further helps cancer to spread to the surrounding cells, by producing an ROS (reactive oxygen species) under low pH and hypoxia.
4. Cancer has been referred to as a ‘wound that never healed’ and it has been found to have all features similar to that of inflammation.

   The epithelium is the site for initiation of carcinoma occurring due to genetic alterations, but the tumor progression is stated to originate from the signals in the stromal microenvironment. The cancer cells are found to discharge stroma-altering growth factors such as fibroblast growth factor, VEGF family members, plasma- derived growth factors, interleukins, epidermal growth factor receptor ligands, transforming growth factor β, colony-stimulating factors, and many others. These factors disrupt the tissue homeostasis by causing stromal reactions such as angiogenesis and inflammatory response by acting in a paracrine manner.[13]

   In the case of the macrophages, they undergo differentiation into the tumor associated macrophages or TAMs under the influence of the CAF causing them to release factors such as the VEGF, HGF, MMP2, and IL-8. They are attracted to the tumor region along defined chemotactic gradients. The TAM further enhances the growth and metastasis of the cancer cells.
5. The stromal cells (CAF) enable the cancer cells to be exposed to the growth factors by bypassing the immune surveillance, by secreting protective cytokines, chemokines, and other factors.

Specialized stem cells called as the cancer stem cells (CSC) are found to be produced by the CAF, which is said to play a role in tumor proliferation. How a site or niche, especially for the CSC produced is still an enigma and there have been many studies being conducted to study the formation of these niches. The theory that they arise from the same area as the normal stem cells was the first possibility. The second possibility was of transmission signals that may activate a relatively quiet niche and activate it to produce the stem cells. The ECM may itself produce the niches; this was the third possibility for formation of these

stem cells. The knowledge of the area of niche formation is important to formulate drugs to act against these stem cells and interrupt the cancer growth.[14]

CONCLUSION

The tumor cells and the stromal cells are made up of a heterogeneous cell population as proved by numerous studies that regulate tumor metastasis, survival and cancer growth. These are formed due to the stimulation of the tumorstroma by various factors to make an envoirnment conducive to them.α -SMA-positive 'stress fibers' play major role in the cross-talk between the stroma and the tumor. Hence, anti-cancer strategies are conceivable with the aim to specifically, target this phenotype in the tumorstroma. Such strategies can target the contractile apparatus of the cancer-associated myofibroblast to reduce ECM stiffening, which contributes to the persistence and progression of the tumor.

While concluding we can say that the underlying mechanisms and the role played by CAFs in tumor progression have been somewhat understood. Even so, the origin and exact functions need scrutiny. Drugs have been identified of which some are still in their experimental stages; that would somewhat interrupt the role of CAFs, but on many of them their specificities need to be enhanced. Added understanding of systematic, practical mechanisms and the pathological roles of CAFs in the tumor microenvironment is estimated to lead to a development ofnew approaches for cancer therapy.

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ABBREVIATIONS

1. Cancer associated fibroblasts---------- CAF
2. Epithelial Mesenchymal Transition--- EMT
3. Transforming Growth Factor-beta----- TGF-β
4. Mesenchymal bone derived stem cells-- MSCs
5. Matrix metalloproteins----------MMP
6. De-oxyribonucleic acid----------DNA
7. Smooth muscle actin----------SMA
8. Fibroblast stimulating protein-1--------FSP-1
9. Platelet-derived growth factor α--------PDGFR-alfa
10. Platelet-derived growth factor-beta-----PDGFR-β
11. Largest subgroup of the Ca^{2+}-binding EF-hand (helix E-loop-helix F) protein group.--S100A4

12. Hepatocyte growth factor----------HGF
13. Human adipocyte stem cells---------- hASCs
14. Stromal-cell-derived factor 1----------SDF-1
15. Regulated on activation, normal T cell expressed and secreted)/ Chemokine Ligand 5 ----------CCL5/RANTES
16. Fibroblast specific protein-1----------FSP1
17. Platelet endothelial cell adhesion molecule ------CD31/PECAM
18. Thrompospondin-1----------TSP-1
19. Tissue inhibitors of MMPs----------TIMPs
20. Zinc finger protein SNAI1 ----------Snail
21. Zinc finger protein SNAI2---------- Slug
22. Zinc finger E-box binding homeobox 1---ZEB-1 and -2
23. Forkhead box Protein----------FoxC3
24. Subfamily of the Ras superfamily -------Rho-family GTPases
25. Stromal cell-derived factor 1 ----------SDF-1α
26. Wingless in Drosophilia---------- Wnt 5
27. Bone Morphogenetic Protein----------BMP 4
28. Tumor associated macrophages----------TAMs
29. Hepatocyte growth factor---------- HGF
30. Cancer stem cells---------- CSC

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