33rd Annual International Conference of the IEEE EMBS
Boston, Massachusetts USA, August 30 - September 3, 2011

Effects of radiotherapy fractionation on breast stromal activity

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Abstract — **We study the dynamics of tumor cell progression as growth factors and ionizing radiation (IR) combine to modify cellular microenvironments. Breast tumor growth depends on the behavior of cancer cells in their microenvironment, and both components are affected by IR fractionation parameters. TGF-β1 promotes differentiation of fibroblasts to myofibroblasts, which stiffens the extracellular matrix (ECM) and promotes malignant cell phenotypes. IR generates reactive oxygen species (ROS) that damages and inactivates cells thus controlling proliferation. The effects of TGF-β1 and IR at various fraction sizes on ECM stiffness and fibroblast differenation are studied using MRC-5 fibroblasts in 3-D collagen cultures.**

I. Introduction

Cancer research has taken a cell centric view when searching for improved diagnosis and treatment. However, there is an emerging role of the biomechanical microenvironment associated with stromal components in predicting tumor progression. This has emphasized the need to monitor stromal involvement when developing therapeutic strategies. Consequently we have constructed a 3D model to isolate stromal components to investigate their role under ionizing radiation (IR) [1].

The microenvironment consists of soluble and insoluble proteins, which are important in system homeostasis [2]. Fibroblasts produce soluble paracrine growth factors that regulate cell proliferation, morphology, survival and death. The insoluble proteins, such as collagen make up the extracellular matrix (ECM). The structure and attachment sites that exist within the ECM constitute the mechanical forces cells experience.

The forces on cells are important in driving disease [11]. Structural integrity, quantified by stiffness, contributes to the force [13]. The stroma is responsible for mechanical changes. ECM components contribute to the stiffness that occurs with tumor progression. It has been shown that the arrangement, structure and degree of crosslinking dictates the mechanical properties of the ECM and controls how mechanical forces are transmitted to cells. The two most abundant stromal components active near a tumor mass are collagen and fibroblasts.

Despite considerable advancement in our understanding of the biology of tumors, the most efficient way to cure cancers still consists of destroying as many abnormal cells as possible. Treatments are surgery, chemotherapy, growth factor inhibitors and radiation therapy. Our treatment of interest is Radiation therapy (RT) which uses IR to destroy abnormal cells.

Altered fractionation for whole breast irradiation has been extensively studied over the past 20 years [3], with recent updated publications from several landmark randomized trials. Recent trials justify the routine use of modest adjustments for adjuvant whole-breast radiotherapy in women with early breast cancer.

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II. Material and Methods

Stromal components, such as collagen I, fibroblasts, and growth factor TGF-β1 have been used as surrogates of native 3D ECM to more accurately model the mesenchymal 3D environment in vivo. We propose a novel approach that mechanically explores fractionation schemes; we studied whether collagen crosslinking could stiffen the ECM through radiation to study radiation treatment protocols. Optimum fractionation in radiotherapy occurs when tumor control is improved without enhancement of complications. Standard curative schedules of radiotherapy to the breast deliver 25 fractions of 180 cGy over 5 weeks. We tested whether altered fraction size would result in similar matrix behavior as the standard protocols. In this analysis, we assessed stiffness and differentiation of 3D collagen gel samples with MRC-5 fibroblast cells with the use of TGF-β1 and ionizing radiation stimulation to mimic cancer progression and treatment. Samples were exposed to high energy X-rays from a linear accelerator to match the irradiation exposure during radiotherapy.

A. Cell Culture

Lung fibroblast cell line MRC-5 were purchased from American Type Culture Collection (Rockville, MD) and cultured in 75cm^2 tissue culture flasks with Minimum Essential Medium Eagle (MEME) additional 10% fetal bovine serum (FBS) and 1% pen/strep at 37°C with 5% CO_2. The cells were harvested from cell culture with 0.25% trypsin/1mM EDTA. Trypsin was neutralized with 10% FBS MEME. The cells were grown to 80-90 percent confluence before use, with average pass number in the range of 5-12.

B. Ionizing radiation

Samples were radiated with a linear accelerator (Varian Medical System, Palo Alto, CA) using 6 MV photon energy. In order to deliver uniform dose to each sample, radiation was delivered with the gantry set to 0 degrees and dosed to dmax of 100 cm source to axis distance. An 8x8-cm^2 field

size was used to ensure that the irradiated cells were well within the field.

C. Mechanical Testing

Once the full dose was delivered samples were then tested under compressive indentation experiments using a TA.XT Plus Texture Analyzer System and a 1 kg load cell (Texture Technologies Corp., Scarsdale, NY) apparatus in our laboratory. The experimental protocol used in this study is the same as that used in our lab's earlier studies on gelatin hydrogels [4]. After removal of excess medium from the top of the sample, a 5 mm diameter spherical indentor was pressed into the surface of the sample at a controlled displacement rate of 10^{-1} mm/min to a final displacement, d, of 2.5 mm.

D. Immuno-Histo Chemistry Analysis

After mechanical measurements, 3D collagen culture samples were fixed and mounted on coated slides, deparaffinized in xylene and rehydrated in a serial dilutions of ethanol. They were then stained with Hematoxylin and developed in water. They were then stained with Eosin, and dehydrated in ethanol and xylene. Hematoxylin Eosin (H&E) images were taken with a Fluorescence Microscope (Zeiss Axiovert 200M Microscope), with a 20X color axio camera.

Along with H&E staining 3D collagen samples were fixed for Immunofluorescence (IF) staining. Immunofluorescence (IF) stained samples were examined with a Leica SP2 laser scanning confocal microscope (Leica, Heidelberg, Germany) with Hg lamp and helium/neon laser and the supplied software (Leica Confocal Software Version 2.00). 488nm excitation wavelength was used for Fluorescein isothiocyanate (FITC) and 633 nm excitation wavelengths were used for To-pro 3.

E. Quantification of α-SMA

Phenotypically, activated fibroblasts are characterized as expressing large amounts of α-Smooth Muscle Actin (α-SMA). To observe differentiation of fibroblast cell, Immunofluorescence (IF) staining was done. Confocal imaging resulted in number of images that required α-SMA-stained cells and nuclei counting to quantify activation ratios. Here, we developed an automated segmentation method to recognize and count two different types of staining from a confocal image. The percentage of cells expressing α-SM Actin was determined by a computerized Count Activated Cells Thresholding image analysis program, developed by our lab. It was used to maintain a constant threshold for determining positive differentiation of cells, represented in Figure 2. In order to count the total cell number and activated cell number, we detected red fluorescence regions (nuclei, representing total cell number) and green fluorescence regions (α-SMA, representing activated cell number) in the images from confocal microscope. The activation ratio was defined by the following equation.

$$Activation\ ratio = \frac{Number\ of\ Green\ fluorescene\ (\propto -SMA)}{Number\ of\ red\ nuclei\ \cap Number\ of\ Green\ fluorescence}$$

Where Number of Green fluorescence represents α-SMA and the denominator indicates the total number of red nuclei and intersection of green cell bodies.

F. Statistical Analysis

By using the inference for means and comparing two samples the power for specified sample size (n=6) for indentation tests and (n=4) for the activation of fibroblast was 1.00, which is sufficient based on the mean difference seen from the different samples tested. Data are represented as mean standard error. Comparison between control and irradiated samples were analyzed by Student's t-test under the approximation of normal distribution. Based on the distribution and the observed test statistics the p-value was computed accordingly and a level of signfiance values: $P \leq 0.05$ was used.

III. Results

A. Ionizing radiation induced stiffness and α-SMA expression

Ionizing radiation produces reactive oxygen species (ROS) in the intra and extracellular water by radiolysis. Energy is released from free radicals, which possess an unpaired electron in their outer orbital, after coming to rest. It has been proposed that cross-linking occurs through energy absorption by aromatic groups within collagen molecule. X-rays degradation of collagen through peptide bond cleavage, however intermolecular crosslinking formation dominates due to fluid content in our samples.

We irradiated MRC-5 fibroblasts seeded in 3D collagen lattices, sample irradiation was 90, 180 or 360 cGy per day for three days for a total dose of 270, 540 or 1080 cGy, and measured mechanical stiffness at 24, 48 and 72 hours.

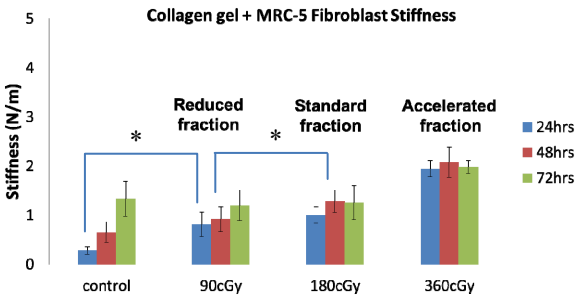

Fig. 1 Irradiation of 3-D collagen cultures with fibroblast cells by therapeutic doses initially increases the net collagen matrix stiffness more than collagen alone (33% at 48 hrs) from control versus 90 cGy. (n=6) Mean±SD. *p<0.05.

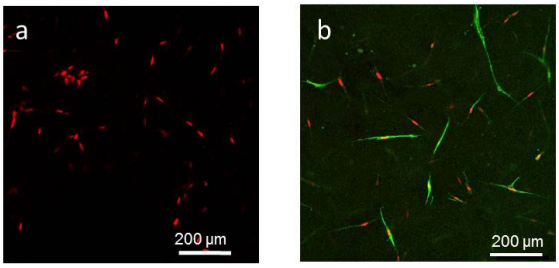

Fig. 2 IHC staining image for (a) unirradiated sample and (b) 180 cGy irradiated sample at 48 hours time point. α-SMA is shown in green and nuclei are in red. Cells with green are activated.

A more pronounced radiation-induced effect was recorded when the dose was given in higher fraction of 360 cGy (Fig 1). In this group of gel samples, the mean stiffness was significantly higher than in all other groups of gel samples treated with radiation. There was a significant difference between un-irradiated samples and irradiated samples. This also correlates to the finding by Wrobal, L.K. el, that stiffer the environment the more contraction force by fibroblast, as seen from the Figure 1 with higher the radiation dose the more cross-linking seen with collagen gels alone. Increasing radiation dose caused a greater stiffness, initially at the 24 and 48 hour time points. However the stiffness at 72 hours drops off. This may be due to IR introducing a block of cells in G1 and G2 cell cycle phase which leads to a synchronization.

Recent evidence suggests radiation causes the differentiation of fibroblasts to myofibroblasts [5], that the cytokine TGF-β1 may be a mediator of radiation-induced terminal differentiation [6]. Dose-dependent activation of latent TGF-β1 has been demonstrated after doses of IR in the range 0.1±5 Gy and reactive oxygen species (ROS) are efficient activators of TGF-β1 [7]. Radiation induced ROS production is important known effects on cell cycle progression and differentiation [8]. This may be due to the difference seen between the 90 cGy and 360 cGy samples.

A molecular marker characteristic of myofibroblasts is Alpha Smooth Muscle Actin (α–SMA). We found that TGF-β1 can be produced by unirradiated as well as by irradiated fibroblasts. This was confirmed by α–SMA staining of the samples with fibroblast and irradiation as seen in Figure 2.

B. Characterization of cell response to TGF-β1 and IR stimulation

TGF- β1 has been known to promote differentiation of fibroblasts to myofibroblasts, which stiffens the extracellular matrix (ECM) and promotes malignant cell phenotypes [9]. It is reported that TGF-β1 would increase generation of contractile force in 3D collagen gel matrix due to cell differentiation upregulating α–SMA [9]. We found that TGF-β1 at 10 ng/ml increased stiffness of myofibroblasts in collagen lattices after 48 hour period without radiation (Fig. 3). These results demonstrate that TGF-β1 can increase myofibroblast contraction of collagen lattices leading to the stiffness increase seen in Figure 3 of control after 72 hours. We examined three different fractionation schemes on gel matrixes. All showed increased stiffness in response to TGF-β1; however, the percentage increase in stiffness varied between the 90cGy fraction and the other two protocols, 180 and 360cGy (Table 1).

There was a greater deal of significance for non-irradiated samples compared to radiated ones. However, moving from the 180 to 360 cGy there is not a great deal of significant difference indicated on Figure 3. Indicating that using a larger dose fraction would not lead to greater stiffness.

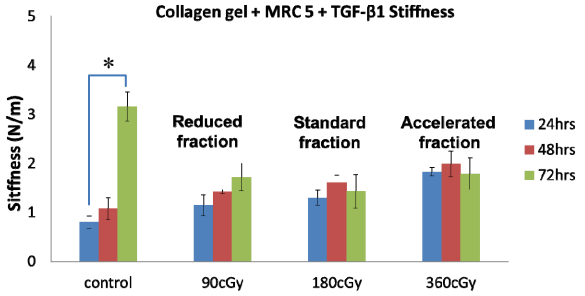

Fig. 3. Irradiation of 3D collagen with TGF-B1 stimulated fibroblasts increases matrix stiffness in the first 24 hours compared to cultures without irradiation. However, higher radiation dose leads to softening of 3D matrix. (n=6) Mean±SD. *p<0.

Table 1

	24 hours	48 hours	72 hours
Control	0.80 (±.13) N/m	1.08 (±.22) N/m	3.16 (±.25) N/m
90 cGy	1.15 (±.21) N/m	1.42 (±.04) N/m	1.72 (±.28) N/m
180 cGy	1.30 (±.15) N/m	1.61 (±.14) N/m	1.43 (±.34) N/m
360 cGy	1.83 (±.08) N/m	1.99 (±.26) N/m	1.79 (±.33) N/m

Table 1 Mechanical stiffness of all 3D collagen matrix gels stimulated by TGF-β1 and Ionizing radiation

We demonstrate that TGF-β1 and ionizing radiation enhanced the formation of the structural elements important in myofibroblast contractile force generation and transmission, through α-SMA expression before 72 hours. The distribution of actin has been determined throughout these experiments in an attempt to delineate the roles of differentiation in stiffness and remodeling process.

C. Correlation between α-SMA expression and stiffness response

In most normal tissues fibroblasts exhibit only immature if any stress fibers (Fig. 4). Increasing matrix tension leads to the formation of the proto-myofibroblast, characterized by mature adhesions and contractile stress fibers that are composed of cytoplasmic actins. The contractile force exerted by stress fibers further augments tension in the matrix, promoting gradual supermaturation of specialized focal adhesions (FAs) [10]. The concerted action of tension and TGF-β induces expression of α-SMA, which organizes transitorily in the cytoplasm.

The presence of microfilament bundles or stress fibers; microtubules and intermediate filaments have recently been documented also in situ [10]. Furthermore, many

myofibroblast cells are highly elongated, exhibit an abundance of oriented microtubules and microfilaments (Fig. 2b). Based on these structures and variety of data, the contractile behavior of the myofibroblast has been seen to be responsible for generating the large stiffness seen.

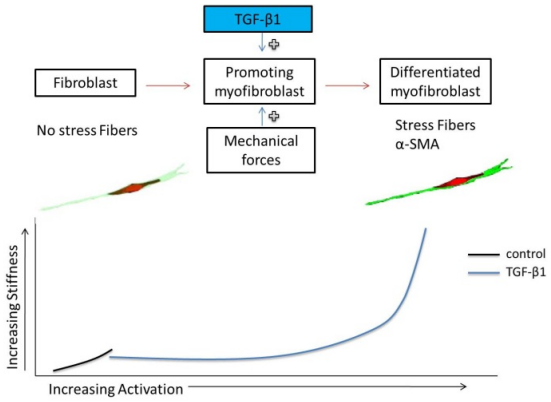

Fig. 4 Correlation between α-SMA expression and stiffness. Normal tissue fibroblasts lack mature stress fibers. However, increasing matrix tension and external stimulation such as TGF-β1 lead to mature stress fibuers and expression of α-SMA.

IV. Discussion

Mechanical properties of the microenvironment are sensed by integrin family receptors that connect ECM proteins outside the cell to the actin cytoskeleton inside the cell [11]. Increased tension from a stiffer matrix induces integrin clustering, the development of focal adhesions, and the activation of multiple downstream signaling pathways, leading to ECM remodeling as seen in tumor progression. We were able to connect the chemical signal with the ECM changes through our measurements of stiffness and cell differentiation. Investigators [12] have showed that tumors developing in stroma that contains LOX crosslinked collagen display elevated integrin signaling, including phosphorylation of focal adhesion kinase (FAK) and Cas. FAK phosphorylation is known to signal the Rho pathway, which influences cell contractility by mediating cytoskeletal processes such as focal adhesion and actin stress fiber formation [13]. They also found that the PI3-kinase substrate AKT is hyperactivated in stiffer tumors. From our findings there may be an intersection between mechanotransduction pathways with oncogenic signaling pathways.

We would like to point out collagen crosslinking and CAFs contraction in the microenvironment as a contributor to stromal stiffening that leads to breast tumor progression. This work also promotes an understanding of cancer not only as, dysregulated cancer centric cellular signaling and behaviors, but also as an ECM dependent pathology where cells interact with and respond to their physical microenvironment. We can see that normal cells respond according to changes that we have administered, be it radiation or TGF-β1. This indicates that carcinomas of the breast could be more effectively treated with larger fraction size than previously thought, possibly without the risk to impair the ratio of local cure to late normal tissue complications. Our data shows that stromal components may be better able to adhere to the notation that a stiffer environment and softening of surrounding tissue may increases understanding of metastatic progression.

V. Conclusion

Tumors are characterized by extracellular matrix (ECM) remodeling and stiffening [12]. Our experimental results both improve our basic understanding of tumor mechanobiology as well as identify relevant therapeutic response. Our approach, for the first time, mechanically explored fractionation schemes; we studied whether collagen crosslinking could stiffen the ECM through radiation to study radiation treatment protocols. Breast cancer tissue has been found to be just as sensitive to fraction size as dose-limiting healthy tissues [3]. These findings are a confirmation; radiotherapy schedules can be evaluated through stiffness and differentiation changes. CAFs and factors they produce are important targets for radiation therapy and prove to be useful prognostic markers.

Acknowledgment

The authors gratefully acknowledge Dr. Masab Garada and Provena Covenant Medical Center for use of facility for radiation work. Dr. Rohit Bhargava along with Jing Xu for contribution to the cell activation work.

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