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Abstract

We studied two human mammary carcinoma cell lines, MCF-7(NEO-4) HER-2 negative and MCF-7(HER-2) HER-2 positive, in three-dimensional culture. The aim of the study was to determine the glycosaminoglycan (GAG) concentration in breast cancer cell growth ex vivo. We found that GAG concentration produced by both breast cancer cell lines can be measured with high-field magnetic resonance imaging.

Keywords Breast cancer cells - Glycosaminoglycan - Magnetic resonance imaging

Introduction

Extracellular matrix (ECM) plays a critical role in cell fate and function (Flaim *et al*., *2005*). The reason for failure of cancer chemotherapeutic treatment is inadequate drug delivery through the cellular microenvironment, which creates a barrier to therapeutic agents (Jain, *1994*; Jang *et al*., *2003*). Therefore, study of the ECM could provide valuable information on tumor development and for drug discovery (McNeish, *2004*).

The ECM is a heterogeneous collection of covalent and noncovalent molecular interactions between primary protein and glycosaminoglycan (GAG). GAG forms a long carbohydrate in breast cancer tissue (Everts and Buttle, *2008*) and due to its negative charge attracts ions (Mareel, *1979*). It was shown that cancer cells developed negative charge in response to biochemical changes in the cellular microenvironment (Muir, *1983*). In breast pathogenesis quantitative changes in GAG may have important consequences on cells' proliferation and drug delivery (Eschenko *et al*., *2007*). Considering the importance of GAG monitoring in small tissue samples (Ling *et al*., *2008*) several techniques have been used to measure GAG content in vitro. These methods are based on high-performance liquid chromatography (HPLC) (Studelska *et al*., *2006*), mass spectrometry (MS) (Harvey, *2006*), electrophoresis (Jaffe, *1977*), and immunofluorescence (Altman *et al*., *1993*). Yet, to provide another method, we studied the ECM using contrast-enhanced $Gd(DTPA)^{2-}$ magnetic resonance imaging (MRI). In our study we investigated GAG content in three-dimensional (3D) ex vivo breast cancer cell cultures using measurements of proton (^1H) T_1 relaxation time. Penetration of $Gd(DTPA)^{2-}$ into cancer cells was monitored through T_1 calculated images obtained after injection into 3D culture.

Two-dimensional (2D) breast cancer cell cultures have been used for many years for in vitro applications. However, 2D culture does not reveal function of cells observed in a 3D culture (Abbot, *2003*) or a concentration which is high enough to be able to detect them by MRI (Sawyer *et al*., *2008*).

Breast cancer cells are very sensitive to changes in their microenvironment and behave more like their in vivo counterparts when they are in 3D culture (Abbot, *2003*). It is also recognized that cellular behavior of 3D cultures is closer to the natural physiology of tumors (Abbot, *2003*). In addition, 3D ex vivo systems consisting of breast tumor cell lines such as MDA-MB-231 have also been shown to recapitulate the drug sensitivities of tumor cells grown in vivo (Debnath *et al.*, *2003*).

It has been established that 3D culture in a hollow-fiber bioreactor simulates in vivo an environment which is favorable for tissue growth and simulates particularly well the spatial organization of solid tumors (Malone *et al.*, *2001*). The bioreactor utilizes hollow fibers to deliver nutrients and to remove waste from an agitated cell suspension. Furthermore, hollow-fiber bioreactors, unlike other cell culture techniques, provide a porous support for cell attachment and resemble in vivo conditions.

To this day there is no generally applicable direct GAG mapping technique available (Ling *et al.*, *2008*), despite the high GAG content in breast cancer tissue (Delehedde *et al.*, *2001*). Thus, for MRI study, we used 3D high-density cell cultures.

Materials and methods

3D breast cancer cell cultures

We used two breast cancer cell lines, MCF-7(HER-2) and MCF-7(NEO-4), obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). All compounds for cell culture were supplied by Fisher Scientific (Ottawa, ON, Canada). Both cell lines were cultured in RPMI-1640 medium, 5% fetal bovine serum, 2 mM L-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin. All cell culture procedures were conducted in a sterile level II safety cabinet. Cells were maintained in tissue culture flasks and were cultured as a monolayer prior to seeding in the hollow-fiber bioreactor (FiberCell Systems Inc., Frederick, MD, USA). When the number of cells in the culture flask reached 0.5×10^7 cells/ml, the culture was harvested and then inoculated into the hollow-fiber bioreactor. The hollow-fiber bioreactor consists of porous hydrophilic hollow fiber of 0.1-µm pores placed in polysulfone tubing. We used a collagen solution to create an ECM between cells and fiber. The polysulfone fiber was coated with protein by flushing with 10 ml of coating solution containing 1 mg collagen per 1 ml phosphate-buffered saline (PBS). After the inoculation, the hollow-fiber bioreactor was perfused using a peristaltic pump. The flow of medium started at the rate of 5 ml/min and was gradually increased to 14 ml/min. The pH in the extracapillary space was maintained at between 6.8 and 7.0 throughout the experiments. The perfusion medium was changed weekly when the glucose level reached 2 g/l measured by glucometer. Breast cancer cells were allowed to grow in the hollow-fiber bioreactor until their density reached 10^9 cells/ml. The number of cells was determined using the Trypan blue (Sigma-Aldrich, Oakville, ON, Canada) exclusion method (Takahashi and Loo, *2004*). Additionally, we used glucose monitoring to count the cells, assuming that the consumption of 1 g of glucose per day corresponds to 10^9 cells inside the hollow-fiber bioreactor (Kirstein *et al.*, *2006*). Eighteen hollow-fiber bioreactors were used for the study: six for controls, MCF-7 (HER-2), and MCF-7(NEO-4), respectively.

Magnetic resonance imaging

Throughout the MRI experiments, hollow-fiber bioreactors were maintained under incubator-like conditions (37°C, 5% CO_2, and 95% air). All MRI experiments were performed using a 9.4-T, 21-cm-bore magnet (Magnex, UK) and TMX console (NRC-IBD, Canada). Water proton longitudinal (T_1) relaxation times of both cell lines were measured using an inversion recovery (IR) pulse sequence with an cho time (TE) of 16.5 ms, a repetition time (TR) of 8000 ms, and eight inversion times (TIs)—10, 100, 200, 400, 800, 1000, 2000, and 4000 ms. T_2 relaxation times were measured using a multi-echo spin-echo (SE) pulse sequence with a TR of 8000 ms and 11 echoes 10 ms apart, with the first TE 16.5 ms. The imaging plane was perpendicular to the long axis of the bioreactor. The field of view was 3 × 3 cm, the slice thickness 2 mm, and the matrix size 256 × 256.

Glycosaminoglycan

The GAG concentration was calculated based on the fixed charge density (FCD) value (Donahue *et al*., *1997*), which was measured by flushing the culture with Gd(DTPA)$^{2-}$ (Berlex, USA). The FCD can be expressed as

$$FCD_{\text{tissue}} = -2\left[Na^{+}\right]_{\text{bath}}\left(\sqrt{\frac{\left[\text{Gd}\left(\text{DTPA}\right)^{2-}\right]_{\text{tissue}}}{\left[\text{Gd}\left(\text{DTPA}\right)^{2-}\right]_{\text{bath}}}}-\sqrt{\frac{\left[\text{Gd}\left(\text{DTPA}\right)^{2-}\right]_{\text{bath}}}{\left[\text{Gd}\left(\text{DTPA}\right)^{2-}\right]_{\text{tissue}}}}\right) \quad (1)$$

where

$$\left[\text{Gd}\left(\text{GDPA}\right)^{2-}\right]_{\text{tissue}} = \frac{1}{R}\left(\frac{1}{(\text{post Gd})T_{1(\text{tissue})}}-\frac{1}{(\text{pre Gd})T_{1(\text{tissue})}}\right) \quad (1a)$$

and

$$\left[\text{Gd}\left(\text{DTPA}\right)^{2-}\right]_{\text{bath}} = \frac{1}{R}\left(\frac{1}{(\text{post Gd})T_{1(\text{bath})}}-\frac{1}{(\text{pre Gd})T_{1(\text{bath})}}\right) \quad (1b)$$

where bath is the medium around breast cancer cells; R, the relaxivity (mmol/l/s); tissue, breast cancer cell tissue; $[Na^{+}]_{\text{bath}}$, the concentration of Na^{+} ions in the bath (154 mM); $(\text{post Gd})T_{1(\text{tissue})}$, the T_1 relaxation time of breast cancer cells after administration of Gd(DTPA)$^{2-}$ solution (s); $T_{1(\text{tissue})}$, the T_1 relaxation time of breast cancer cells before administration Gd(DTPA)$^{2-}$ solution (s); $(\text{post Gd})T_{1(\text{bath})}$, the T_1 relaxation time of the bath after administration of Gd(DTPA)$^{2-}$ solution (s); and $T_{1(\text{bath})}$ the T_1 relaxation time of the bath before administration of Gd(DTPA)$^{2-}$ solution (s).

The calculated FCD was converted to GAG concentration according to Lesperance *et al*., (*1992*):

$$\text{GAG} = \text{FCD}\left(502.5/-2\right) \quad (2)$$

where GAG is the glycosoaminoglycan concentration (mg/l), the FCD is millimolar concentration; and 502.5 is the molecular weight of GAG (mg/mmol).

To measure GAG concentration, $Gd(DTPA)^{2-}$ solution was injected into the bioreactor perfusion tubing flow of the medium. The injected volume was calculated to give the final concentration 2 mM $Gd(DTPA)^{2-}$ (Donahue *et al.*, *1994*). Medium with $Gd(DTPA)^{2-}$ was circulated constantly through the bioreactor using the peristaltic pump. Constant T_1 was achieved 24 h after the injection of $Gd(DTPA)^{2-}$ solution. For calculations of T_1 relaxation times, MAREVISI (NRC-IBD, Canada) software was used.

Water content

After MRI analysis, 2 mg cells was extracted from the bioreactor and weighted to determine the wet weight. Dry weight was determined by drying the tissue samples at 110°C until constant weight was achieved. The percentage water content was determined from the wet and dry weights.

Data analysis

All data reported here are from sets of six separate experiments. Results are expressed as mean ± standard deviation. Data were analyzed using the Sigma Stat Soft (Chicago, IL, USA) software. The reproducibility of the assay was demonstrated by analyzing with MRI 10 identical samples of the GAG (Sigma) water solution with a concentration of 5 mg/ml. The MRI procedure used exactly the same parameters as for real samples of cells. The GAG solution was introduced to tubes of 50-ml volume and measured at 37°C. Moreover, the reproducibility was determined using two samples of MCF-7 (HER-2) and MCF-7(NEO-4) cells after 7 weeks in culture, and 10 identical MRI measurements were repeated.

Results and discussion

In our experiment, we cultured 3D ex vivo human breast carcinoma cells using a hollow-fiber bioreactor over 7 weeks. The cell concentration provided by the bioreactor was sufficient for MRI study. The total cell content in the hollow-fiber bioreactor increased from 0.5×10^7 to 1×10^9 during culture. The hollow-fiber bioreactor was placed inside the magnet and removed without disturbing the regions of cells. Furthermore, multiple MRI measurements of the same cells were performed.

Over time in culture the water content decreased significantly for MCF-7(NEO-4) and MCF-7(HER-2) cells, from 93 ± 9% to 77 ± 5% and from 89 ± 7% to 68 ± 4%, respectively. The T_1 relaxation time of MCF-7(NEO-4) decreased during the 7 weeks from 2739 ± 189 to 2121 ± 178 ms, and T_2 from 143 ± 14 to 64 ± 5 ms. T_1 measurements of MCF-7(HER-2) showed a decrease from 2574 ± 209 to 1894 ± 299 ms; and T_2 measurements, from 148 ± 19 to 72 ± 17 ms. Values of T_1, T_2, and water content after inoculation are presented in Table 1 for MCF-7(NEO-4) HER-2 negative and MCF-7(HER-2) HER-2 positive, respectively. Correlations between tissue water content and relaxation times T_1 and T_2 were linear (Fig. 1). Changes in

GAG concentration were measured for both cell lines when the number of cells in the culture reached a high density, at weeks 6 and 7. GAG concentrations were 2.86 ± 0.8 and 2.73 ± 0.5 mg/ml, and 1 week later 3.01 ± 0.4 and 2.78 ± 0.9 mg/ml, for MCF-7(NEO-4) and MCF-7(HER-2), respectively (Table 1).

Table 1 MRI tissue parameters and GAG content: mean ± SD

Time (weeks)	T_1 **(ms)**	T_2 **(ms)**	**Water content (%)**	**GAG content (mg/ml)**
MCF-7(NEO-4) HER-2 negative				
1	2739 ± 189	143 ± 14	93 ± 9	–
3	2415 ± 233	114 ± 18	85 ± 10	–
6	2187 ± 221	81 ± 21	79 ± 10	2.86 ± 0.8
7	2121 ± 178	64 ± 5	77 ± 5	3.01 ± 0.4
MCF-7(HER-2) HER-2 positive				
1	2574 ± 209	148 ± 19	89 ± 7	–
3	2443 ± 226	134 ± 11	86 ± 6	–
6	1919 ± 238	122 ± 21	73 ± 11	2.73 ± 0.5
7	1894 ± 299	72 ± 17	68 ± 4	2.78 ± 0.9

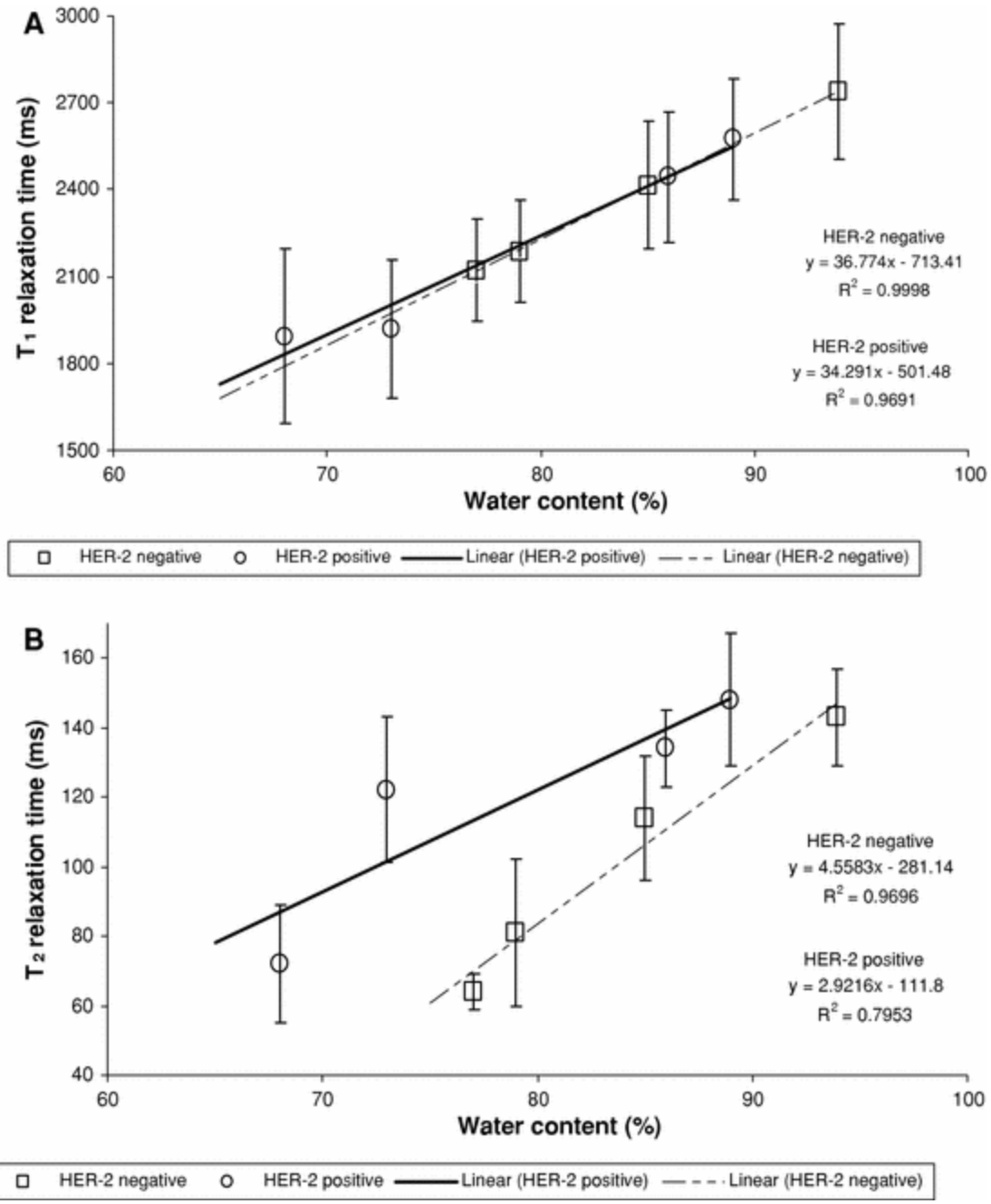

Fig. 1 T_1 (**a**) and T_2 (**b**) of breast cancer cell lines

MCF-7 (NEO-4) cells measured for reproducibility after 7 weeks in culture showed GAG concentrations with a mean value of 2.74 mg/ml and a standard deviation of 0.26 mg/ml. For a sample of MCF-7 (HER-2) cells the mean GAG concentration was 2.53 mg/ml, with a standard deviation of 0.25 mg/ml. Samples with a known GAG concentration of 5 mg/ml, considered in the reproducibility test, were found to have GAG concentrations ranging from 4.52 to 4.91 mg/ml, with a mean value of 4.76 mg/ml and a standard deviation of 0.25 mg/ml.

Figure 2 shows an example of a proton MR image of the bioreactor with a fiber. The results of the study demonstrated that the GAG concentration in human breast carcinoma cells can be measured and quantified using ex vivo MRI. In addition, GAG concentrations determined in our study correspond to the concentration measured in human breast cancer tissue, which is ≥5 mg/ml (Marotta *et al.*, *1985*). It was found previously that proton T_1 relaxation time depends on tissue composition (Chatell *et al.*, *1986*) and that the decrease in T_1 in carcinoma cell cultures is caused by the decrease in water content and increase in macromolecular matrix components (Stack *et al.*, *1990*). We contribute the changes in T_1 and T_2 during cell growth observed for both cell lines to the changes in tissue hydratation and protein content. In addition, our study showed that proton T_1 and T_2 relaxation times are not significantly different between the two cell lines.

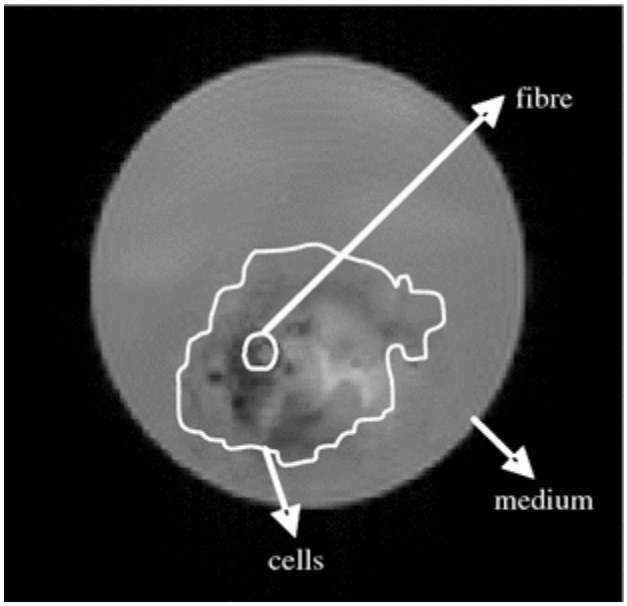

Fig. 2 Proton MR image. MCF-7 breast cancer cells surround the fiber (6 weeks after inoculation). A spin-echo pulse sequence was used, with the following parameters: TR/TE, 8000/16.5 ms; FOV, 3 × 3 cm; matrix, 256 × 256; and slice thickness, 2 mm. The *solid line* delineates tumor cells

Application of MRI to study of cancer cells has several significant advantages over chromatographic, mass spectrometric, and electrophoretic methods because it enables direct study of cells before and after treatment, avoiding the problems stemming from chemical derivatization and from pH sensitivity. Moreover, all these methods require special preparation of the sample to make the compound detectable and to increase the efficacy of measurements. However, MRI can be used in diagnostic applications to continuously monitor amounts of GAG in cell culture without preisolation and special preparation. MRI assay is performed in one step and on live cells during measurements and can provide more information on the physiology and metabolism of cancerous cell cultures. The T_1 and T_2 relaxation times of cells are sensitive to molecular structure and architecture, and have been shown to demonstrate changes in anatomically intact tissue ex vivo. Moreover, MRI measurements of cells with using the anionic paramagnetic contrast agent $Gd(DTPA)^{2-}$ reflect directly the GAG concentration in tissue and are sensitive to physiologic and pathologic conditions, resulting in an approximately linear

relation between GAG content and T_1 relaxation time. The implemented experimental system provided controlled conditions and allowed for reproducible experimental setup, as well as quantification. Moreover, multiple MR measurements of the same cells can be performed, as the entire bioreactor can be placed inside the magnet and removed without disturbing the cells. In this manner, the progress of accumulation of ECM can be observed over the course of time and the consistent changes in water content resulting in changes in T_1 and T_2 values. Moreover, the MRI technique involves cellular administration of the negatively charged contrast agent $Gd(DTPA)^{2-}$, which requires sufficient time to penetrate the high-density cell culture. For correct evaluation of GAG using MRI of cells, T_1 measurements need to be done at the exact time of cell growth. One important restriction with these techniques is that they measure strong interactions of $Gd(DTPA)^{2-}$ with cancer cells and require high cell densities. Since GAGs have negatively charged side chains, $Gd(DTPA)^{2-}$ is distributed at higher concentrations in areas with lower GAG concentrations. Therefore, a low T_1 value after administration of contrast agent indicates a low GAG concentration.

The development of breast cancer cells is very intricate and a better understanding of the roles of many more proteins involved in cancer is still needed. The research presented here provides information on various GAG concentrations but also it might also reveal the barrier of drug delivery to cancer tissue in tumors. Our results show the possibility of using MRI for direct GAG quantification in breast cell cultures. According to our knowledge, these are the first studies of 3D breast cell cultures using high-field MRI to determine GAG concentration.

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