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Determining tamoxifen sensitivity using primary breast cancer tissue in collagen-based three-dimensional culture

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ABSTRACT

We developed a three-dimensional assay prepared from primary breast cancer tissue and quantified tumor response to tamoxifen therapy. Freshly harvested breast cancer biopsies obtained at the time of curative surgical resection were fragmented and embedded into collagen I cushions. Changes in proliferation, apoptosis and tumor volume in response to tamoxifen treatment were quantified using image analysis software and optical projection tomography. Individual and collective invasion of epithelial cells into the surrounding collagen I was observed over the course of the experiment using phase contrast light microscopy and histopathological methods. Addition of tamoxifen to preparations derived from ER+ tumors demonstrated a range of response as measured by proliferative and apoptotic markers. In keeping with published data, tamoxifen reduced the percentage of apoptotic cells expressing cleaved caspase-3 ($p = 0.02$, Poisson regression analysis). Tamoxifen also reduced residual epithelial volume in ER+ tumors ($p = 0.001$, Mann–Whitney test), but not in ER low/- tumors ($p = 0.78$). Changes in tumor volume, as measured by optical projection tomography, allowed stratification into responsive and non-responsive tumors. The model mirrors observations of breast cancer response and histopathological changes to tamoxifen in neo-adjuvant trials. This assay provides a method of screening a battery of therapeutics against individual cancers, informing subsequent design of neo-adjuvant trials.

1. Introduction

Estrogen receptor positive (ER+) breast cancers constitute 70% of invasive breast cancers diagnosed [1]. Targeted anti-hormone therapy is the cornerstone of treatment for these patients, with tamoxifen prescribed for both pre- and post-menopausal women [2]. However, not all patients derive a benefit from these well-established targeted therapies. In one neo-adjuvant trial, 33% ER+ participants demonstrated no clinical response (<25% reduction in tumor burden) to tamoxifen [3]. Moreover, the side-effect profile of tamoxifen is not insubstantial, with increased risk of developing potentially life-threatening complications (relative risk of endometrial cancer 2.70, stroke 1.49, pulmonary emboli 1.88) [4].

Identification of ER+ tumors with tamoxifen insensitivity will allow alternative endocrine therapies to be considered, such as goserelin [5] or fulvestrant [6]. Culture of primary tumor explants in order to determine an individual sensitivity to treatment has been previously reported [7]. Nonetheless, experiments utilising a range of ER+ tumor materials *ex vivo* have consistently underestimated the response to tamoxifen or aromatase inhibitors seen in patients [8–10]. When primary breast tissue orthotopically was engrafted in to nude mice, only 2.5% of ER+ tumours were successfully implanted, compared with a 25% success rate when ER- tissue was used [11].

Despite being the commonest breast cancer sub-type (>70% of patients), only 19 of the 51 cell lines (<40%) described by Neve et al. [12] are ER+. When used in xenografts, ER+ cell lines display a poorly invasive phenotype and rarely metastasize [13]. Nonetheless, response to tamoxifen in ER+ cell line xenografts had some similarities to clinical findings [14,15].

An increasingly well-recognised alternative to the culture of cells, on plastic or in xenografts, is through the use of three-

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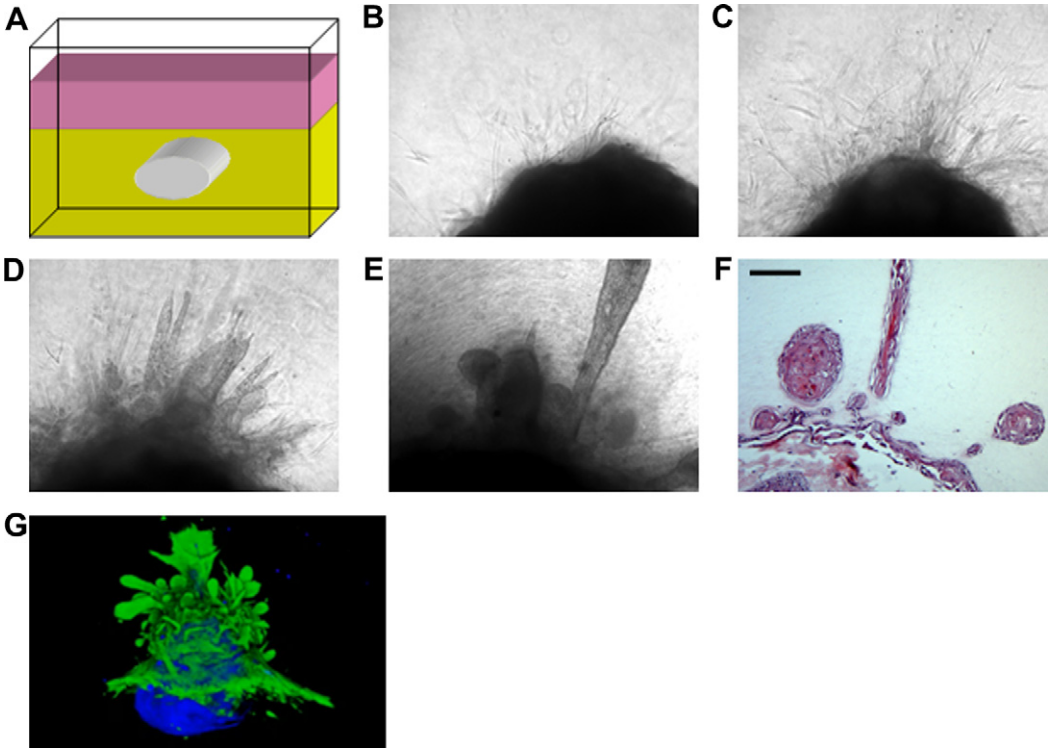

Fig. 1. Three dimensional (3D) culture and tumor *ex vivo* behavior **A** Breast tumor biopsies were dissected into fat depleted fragments (*white*) and embedded in to the centre of Type I Collagen (*yellow*) in a 24 well plate. Mammary Epithelial Growth Media supplemented with β-oestradiol was added on top after a further hour of gelling (*pink*). Brightfield image of tumor edge at **B** Day 8, **C** Day 12, **D** Day 14, **E** Day19. These demonstrate an initial phase of mesenchymal cells invading in to the surrounding collagen followed by collective invasion of small organised structures. These structures extend and mature over the time course. **F** Representative section of assay following fixation and HE staining. **G** Images taken using OPT and Volocity of an assay fixed at day 20. Cytokeratin labels epithelial structures (green) invading from the original core (blue). Bar = 100 μm.

dimensional (3D) matrices [16]. Through 3D techniques, a greater understanding of epithelial organisation and polarity has developed, along with the dynamic reciprocity between epithelium and extracellular matrix [17]. These models have been used extensively to shed light on a number of facets of oncogenesis as well as identification of potential therapeutic targets [18–22]. More recently, collagen-based 3D cultures were developed to include the interaction between epithelial cell lines and primary stroma [23–25]. The complex organisation of such cultures prevented in most cases quantification of cancer cell proliferation, death or invasion [22,26].

Here, we adapted these 3D techniques and the robust measurement and analysis of changes occurring to create an *ex vivo* assay of tumor sensitivity to tamoxifen therapy. This 3D *ex vivo* culture method maintains heterogeneous cell mix of the primary tumor for an extended period and promotes tumor proliferation and invasion [27]. The cultured tumor materials were studied using two quantitative tools. First, immuno-fluorescent images taken from 2D sections were analysed with imaging software, which has been previously validated as a method of quantifying ER and HER2 status comparable to a qualified pathologist [28]. Second, optical projection tomography (OPT) [29] was used, for the first time in 3D cultures, to directly quantify the epithelial volume of the tumor *ex vivo*.

In order to demonstrate tumour sensitivity in 3D *ex vivo* culture, tamoxifen was added to both ER+ and ER- preparations and response has been quantified. The range of responses to tamoxifen seen was comparable to that seen in neo-adjuvant tamoxifen trials. This 3D *ex vivo* assay of breast cancers could potentially provide a method of individualising cancer therapy. This could allow testing an array of therapeutics and quantifying change in biomarkers as well as tumor response *ex vivo*.

2. Materials and methods

2.1. Ethical approval

The use of tissue from invasive breast cancer treated at the Edinburgh Breast Unit at the Western General Hospital was approved by the Lothian Research Ethics Committee (08/S1101/41).

2.2. Three dimensional culture of primary breast cancer ex vivo

Multiple core biopsies were harvested from consenting patients at the time of curative surgical resection for invasive breast cancer. Cores were divided by eye using a scalpel into 1 mm^3 fragments. Macroscopically distinct fat was trimmed and discarded. Tumor samples were then immersed in MEGM Complete (Lonza CC-3150).

To create the collagen gel, 1 mg/ml rat tail collagen, 1:1000 filtered acetic acid, 10x DMEM and 0.22M NaOH were mixed on ice at concentrations of 30%, 50%, 10% and 10% to create a final collagen concentration of 0.3 mg/ml, as used previously [30]. 1.2 ml of collagen mixture was put into individual wells of a 24 well plate and placed into an incubator at 37 °C for 10 min to initiate gelling. After 10 min, tumor samples were placed on to the gelling collagen and pushed in to the centre using a pipette tip. The 24 well plate was then returned to the incubator. MEGM Complete media was supplemented with β-estradiol to create a concentration of 3.2×10^{-10} M. After 1 h, 1.2 ml of the supplemented media was carefully introduced into each well. The final β-estradiol concentration within the assay equilibriated to 1.6×10^{-10} M to recapitulate physiological oestrogen levels found in breast tissue [31]. A pipette tip was run around the inner edge of each well, releasing each gel circumferentially, thus allowing each gel to float freely within the media. Supplemented media was then exchanged twice weekly until termination of the experiment using formalin fixation.

2.3. Tamoxifen treatment of tumor preparations in 3D ex vivo culture

Core biopsies were taken from ten ER+ and five ER- breast cancers undergoing resection with curative intent (Supplementary Table 1). For each patient at least four core biopsies were collected, yielding sufficient material to complete 24 preparations, as described above. Each 24 well plate was inspected on day 7–9. Preparations