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Original Article

Tumor cell culture on collagen–chitosan scaffolds as three-dimensional tumor model: A suitable model for tumor studies

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ABSTRACT

Tumor cells naturally live in three-dimensional (3D) microenvironments, while common laboratory tests and evaluations are done in two-dimensional (2D) plates. This study examined the impact of cultured 4T1 cancer cells in a 3D collagen–chitosan scaffold compared with 2D plate cultures. Collagen–chitosan scaffolds were provided and passed confirmatory tests. 4T1 tumor cells were cultured on scaffolds and then tumor cells growth rate, resistance to X-ray radiation, and cyclophosphamide as a chemotherapy drug were analyzed. Furthermore, 4T1 cells were extracted from the scaffold model and were injected into the mice. Tumor growth rate, survival rate, and systemic immune responses were evaluated. Our results showed that 4T1 cells infiltrated the scaffolds pores and constructed a 3D microenvironment. Furthermore, 3D cultured tumor cells showed a slower proliferation rate, increased levels of survival to the X-ray irradiation, and enhanced resistance to chemotherapy drugs in comparison with 2D plate cultures. Transfer of extracted cells to the mice caused enhanced tumor volume and decreased life span. This study indicated that collagen–chitosan nanoscaffolds provide a suitable model of tumor that would be appropriate for tumor studies.

1. Introduction

Cancer is one of the most important diseases that causes death. For instance, approximately one out of every eight women will be diagnosed with breast cancer [1]. *In situ* evaluation of breast cancer development and metastasis takes 5–30 years. Regarding this point, the development of a suitable model system for use in studying cancer progression at a molecular basis is highly desirable [2].

Current treatments of malignant tumors are usually based on radiotherapy, chemotherapy, and immunotherapy [3]. However, clinical usages of these treatments need to be proven by previously developed screening tests. Development of effective therapies requires a cost-effective *in vitro* tumor

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model that more accurately resembles the *in vivo* tumor microenvironment. Currently, breast cancer screening protocols are done in the two-dimensional (2D) culture of tumor cells [4].

Although 2D culture provides some advantages including accessibility and simplicity, the natural microenvironments are completely different with 2D cultures. In 2D conditions, cell–cell contacts, cell–matrix interaction, cell surface receptor expression, proliferation, and aggressiveness (malignancy) of tumor cells are extensively changed. Furthermore, hypoxic conditions as a typical feature in the tumor microenvironment are rarely provided in 2D cultures. Therefore, outcomes from 2D *in vitro* culture could not translate the exact situation of *in vivo* systems [5–7].

Three-dimensional (3D) culture systems are designed to bridge the gap between *in vitro* and *in vivo* cancer models. These 3D systems mimic extracellular matrix (ECM) conditions and provide a more malignant *in vivo*-like phenotype of tumor cells [8,9]. In addition, it has been reported that tumor cells can show partial differentiation in 3D cultures [10]. At *in vivo* conditions, mechanical and physicochemical support of tumor cells is provided and tumor cells can interact with the ECM. The five classes of macromolecules including collagens, laminins, fibronectins, proteoglycans, and hyaluronans establish the natural ECM. Interstitial spaces are often filled with polysaccharides and fibrous proteins [11,12]. Collagen, as one of the main components of the ECM, plays an important role in supporting and interaction with tumor cells [13]. Spaces between basement proteins are usually filled by polysaccharides. Chitosan is a biodegradable, semicrystalline polysaccharide obtained by N-deacetylation of chitin, which is harvested from the exoskeleton of marine crustaceans and is vastly used for construction of scaffolds and also for drug/gene deliveries [14,15].

Therefore, in the present study we aimed to investigate tumor cell growth rates, resistance to X-ray radiation, and cyclophosphamide based chemotherapy in 2D and 3D cultures. Also, we evaluated *in vivo* tumor growth rate and mice survival as well as systemic immune responses following injection of precultured 4T1 cells to mice in 2D/3D cultures.

2. Materials and methods

2.1. Animals, cell line, and materials

Inbred Balb/c mice (6–8 weeks-old) and documented 4T1 cell line were obtained from the Pasteur Institute (Tehran, Iran). Chitosan (100–300 kDa; 75–85% degree of deacetylation) and 4-hydroxycyclophosphamide as the active form of cyclophosphamide were purchased from Sigma (St. Louis, MO, USA). Flat-bottomed plates were bought form Nunc (Kamstrup, Denmark). Mouse cytokine enzyme-linked immunosorbent assay (ELISA) kits were obtained from eBiosciences (Frankfurt, Germany). RPMI (Roswell Park Memorial Institute) 1640 and fetal bovine serum (FBS) were bought from Invitrogen (Gibco, Grand Island, NY, USA). Collagen Type I was isolated from fresh bovine tendon using a method of trypsin digestion and acetic acid dissolution described previously in Ma et al (2003) [16].

2.2. Preparation of collagen–chitosan scaffold

Collagen or chitosan was dissolved in 1% acetic acid solution to prepare 2% (w/v: 2 g/100 mL) solutions. The chitosan solution was then slowly dripped into the collagen suspension to achieve a final collagen–chitosan ratio of 9:1; thereafter, the materials were homogenized to obtain a collagen–chitosan blend. This blend was then injected into a mold (diameter = 15 mm, depth = 3 mm), frozen at −20°C for 1 hour, and then placed at −70°C. The samples were then dried in a lyophilizer (Edwards MicroModulyo, Bolton, England).

2.3. Cross-linking treatment

Lyophilized scaffolds were rehydrated and stabilized in ethanol. In this process, scaffolds were immersed in absolute ethanol for approximately 1 hour and then sequentially in 70% (v/v) and then 50% ethanol for 30-minute periods. The scaffolds were finally equilibrated in phosphate-buffered saline (PBS, pH 7.4) prior to mechanical testing.

2.4. Scanning electron microscopy of matrices

The matrices were analyzed by scanning electron microscopy (SEM). The sample was coated with a 10-nm thick gold film using a sputter coater, and then analyzed by an electron acceleration voltage of 20 KeV in a DSM 940A SEM system (Zeiss, Hamburg, Germany).

2.5. 4T1 Tumor cell line culture and action

The 4T1 cell line (NBCI code: C604), which mimics Stage IV of human breast cancer, was obtained from the Cell Bank of Pasteur Institute of Iran (Tehran, Iran). 4T1 cells were first cultured overnight in T25 culture flasks and then the cells were recovered and cultured on prepared scaffolds or routine 24-well plates adjusted to 5×10^5 cells/mL RPMI medium [now supplemented with 11mM $NaHCO_3$, 2mM L-glutamine, 100 U penicillin/mL, 100 μg streptomycin/mL, and 5% FBS (all reagents from Gibco)]. For further evaluations, the 4T1 cells were treated with the active form of cyclophosphamide (4-hydroxycyclophosphamide; 1–100 μg/mL) and in another series the cultured cells were exposed to X-ray radiation (0.5–5 Gy). Thereafter, treated cells were incubated for 48 hours at 37°C and under 5% CO_2. After this period, the cells were examined for viability.

2.6. Assessment of cell viability

4T1 cells viability was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay indicative of cellular metabolic activity. At the end of the 48-hour incubation, MTT (20 μL, at 5 mg/mL in PBS) was added to each well and the plate incubated for 4 hours at 37°C. After incubation, culture supernatants were gently removed and 100 μL acidic isopropanol (0.04M HCl in isopropanol) was added in order to dissolve the formazan crystals generated within the viable cells. Absorbance in each well was then assessed at 540 nm using a Multiskan plate reader (Thermo Scientific, Vantaa, Finland). All results were