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Research article

Fibroblast spheroids as a model to study sustained fibroblast quiescence and their crosstalk with tumor cells

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ABSTRACT

Stromal fibroblasts have an important role in regulating tumor progression. Normal and quiescent fibroblasts have been shown to restrict and control cancer cell growth, while cancer-associated, i. e. activated fibroblasts have been shown to enhance proliferation and metastasis of cancer cells. In this study we describe generation of quiescent fibroblasts in multicellular spheroids and their effects on squamous cell carcinoma (SCC) growth in soft-agarose and xenograft models. Quiescent phenotype of fibroblasts was determined by global down-regulation of expression of genes related to cell cycle and increased expression of p27. Interestingly, microarray analysis showed that fibroblast quiescence was associated with similar secretory phenotype as seen in senescence and they expressed senescence-associated-β-galactosidase. Quiescent fibroblasts spheroids also restricted the growth of RT3 SCC cells both in soft-agarose and xenograft models unlike proliferating fibroblasts. Restricted tumor growth was associated with marginally increased tumor cell senescence and cellular differentiation, showed with senescence-associated-β-galactosidase and cytokeratin 7 staining. Our results show that the fibroblasts spheroids can be used as a model to study cellular quiescence and their effects on cancer cell progression.

1. Introduction

In healthy tissue, fibroblasts are maintained in an inactive state and they regulate the proper tissue architecture by controlling the composition of extracellular matrix (ECM). During wound healing and in pathological conditions, such as inflammation and cancer, they become activated and begin to secrete cytokines and growth factors [1,2]. In cancer, these activated fibroblasts (cancer-associated fibroblasts, CAFs) can support cancer progression [1,2], whereas normal fibroblasts have been shown to restrict cancer growth [2,3]. A better understanding of the transition of fibroblasts from quiescence into active state would aid the understanding of cancer progression. Cellular quiescence is not merely a passive arrest in response to nutrient starvation, loss of adhesion or acquisition of confluence, but it seems to be a controlled program with active metabolism and where reversibility is ensured and terminal differentiation is suppressed [4,5].

Nemosis is an experimental model of fibroblast remodeling caused by detaching fibroblasts from their growth support and allowing them spontaneously form multicellular spheroids (reviewed in ref [6]). Nemosis was initially characterized by induction of the stress protein cyclooxygenase-2 (COX-2) [7], an important mediator of inflammation and carcinogenesis [8]. During nemosis the gene expression profile of fibroblasts is altered and they up-regulate expression of cytokines (interleukin (IL)− 1β, IL-6, IL-8, IL-11, granulocyte-macrophage colony-stimulating factor (GM-CSF), leukemia inhibitory factor (LIF), chemokines (MIP-1α, RANTES), growth factors (hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), keratinocyte growth factor (KGF)) and proteases (matrix metalloproteinase (MMP)-1, MMP-10, MMP-14, and plasminogen activation [9–14]. Furthermore, nemotic fibroblasts induce migration of melanoma cells [10] and keratinocytes [15], whereas in leukemia cells they cause proliferation arrest and induce differentiation of c-Met-negative cells to dendritic-like cells *in vitro* [12]. However, the effect of nemotic fibroblasts on tumor growth has not been studied *in vivo*.

In this study, we analyzed phenotype and gene expression profile of fibroblast spheroids and analyzed their effect on the growth of metastatic variant of human keratinocyte-derived HaCaT cell line (RT3) [16] both *in vitro* and in xenograft tumors *in vivo*. We show that fibroblast spheroids enter quiescence and that these quiescent fibroblasts are able to slower tumor growth when tumors are formed in their presence. We also show that fibroblasts spheroids provide a useful model to study sustained quiescence and its cross-talk with cancer cells.

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2. Materials and methods

2.1. Cell cultures

Human foreskin dermal fibroblasts (HFSF) (kindly provided by Dr. Magdalena Eisinger, Memorial Sloan-Kettering Cancer Center, New York), HT-1080 (American Type Cell Culture Collection, Manassas, VA), AT9733 (American Type Cell Culture Collection) and RT3 (kindly provided by Prof. Dr. Petra Boukamp and Prof. Dr. Norbert E. Fusenig, DKFZ, Heidelberg, Germany) cells were cultured in DMEM/F-12 or DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 5% FCS (Invitrogen), 100 μg/ml streptomycin, and 100 U/ml penicillin. Fibroblast spheroid formation was initiated as previously described [7]. In brief, 200 μl aliquots/well of single cell suspensions (5×10^4 cells/ml; 10,000 cell per well) were plated on agarose-pretreated U-bottom 96-well plates (Costar, Cambridge, MA). Only one spheroid was formed per well.

2.2. Soft-agar assay

The soft-agar colony forming assays were performed as previously described [9]. Briefly, for monolayer group fibroblast were seed normally to cell 6-well cell culture plate and allowed to form monolayer over night. On a following morning monolayer was overlayed with 0.5% agarose diluted in growth medium and let to solidify. For spheroid group fibroblast spheroids were mixed in 0.5% agarose diluted in growth medium that was poured to 6-well cell culture plate and let to solidify. Five thousand RT3 cells/well were mixed to 0.3% agarose diluted in growth medium and laid on the top of bottom agarose. Plates were put to cell incubator for 25 days and formed RT3 colonies were photographed and images were analyzed using NIH ImageJ software (http://rsb. info. nih. gov/ij/).

2.3. Immunofluorescence and senescence-associated β-galactosidase staining

Spheroids were collected at the indicated times and embedded in Tissue Tek OCT (Sakura Finetek, Tokyo, Japan) or paraffin. Immunohistochemistry was performed using the Ventana Discovery immunohistochemistry Slide Stainer (Ventana Medical Systems, Tuscon, AZ). Senescence-associated-β-galactosidase (SA-β-gal) activity was detected as described [17]. The results were interpreted by three experienced users.

2.4. Immunoblotting

Samples were run in 8–20% gradient SDS-PAGE, transferred to nitrocellulose membranes, and incubated with indicated antibodies. Immunoreactive proteins were visualized with the appropriate primary and secondary antibodies using ECL detection (Pierce, Rockford, IL). Densitometric analysis of autoradiographs was performed using the NIH ImageJ software.

2.5. FACS

Cell were fixed with 70% ethanol, washed and stained with propidium iodide (10 μg/ml) (Invitrogen) and analyzed with FACScanner (BD Biosciences, San Jose, CA).

2.6. RNA isolation and microarray analysis

RNA for the microarray analysis was extracted using Trizol Reagent (Invitrogen) and purified with the RNeasy kit (QIAGEN). HG-U133A GeneChips (Affymetrix, Santa Clara, CA) were processed according to manufacture's instructions. Data were analyzed with Affymetrix® Microarray Suite 5.0 and GeneSpring™ software (Agilent Technologies, Palo Alto, CA).

2.7. Xenograft model

In order to generate tumors, RT3 cells alone, with fibroblasts or with fibroblast spheroids were mixed with growth factor reduced Matrigel (BD Biosciences), and injected subcutaneously in the abdominal area of Balb/c nude (Scanbur, Sweden) or NOD/SCID (Charles Rivers Laboratories International, Wilmington, MA) mice. Tumor growth was monitored by measuring the volume using a manual caliber. All animal experiments were performed according to the guidelines approved by the ethics committee of the National Laboratory Animal Center Finland (License number STU 237 A/ESLH-2006-00185/Ym-23, Kuopio, Finland).

3. Results

3.1. Nemotic fibroblasts suppress the growth of human keratinocyte-derived RT3 cells

We have previously shown that the nemotic fibroblasts enhance migration of RT3 cells, a metastatic variant of the human keratinocyte-derived HaCaT cell line [16], through soluble mediators [14]. Therefore, we investigated the effect of nemotic fibroblasts on the growth of RT3 cells in more detail by using a soft-agar assay. Cells were seeded in the top agar and different amounts of fibroblast spheroids (96 or 180, each spheroid contained approximately 10,000 cells) were placed in the bottom layer. As a control, we plated different amounts (0, 50,000, 500,000) of fibroblasts grown as monolayer under the bottom agar. During the experiment (25 days) monolayer control cells grew until they reached confluency (approximately 800,000 cells/well). Fibroblast monolayer supported the growth of RT3 cell colonies and resulted significantly larger colonies. Small number of fibroblast spheroids (96) similarly enhanced the growth of RT3 cell colonies, whereas higher number of the fibroblast spheroids (180) reversed the enhancement effect and resulted colonies similar in size as the RT3 cell colonies growth without fibroblasts (Fig. 1(A)). Fibroblast spheroids also changed the shape of RT3 colonies from round to irregular while the RT3 colonies grown on top of the monolayer fibroblasts or without fibroblasts (control group) remained round. These irregular colonies also appeared to be decomposed (Fig. 1(B)).

To address the effect of quiescent fibroblasts on tumor growth *in vivo*, we implanted RT3 cells alone, with the monolayer-cultured fibroblasts or with fibroblast spheroids into subcutaneously immunocompromised nude mice. We monitored the tumor growth by measuring the tumor volume twice a week for 34 days. Similar to the soft-agar assay, fibroblast spheroids inhibited the growth of RT3 tumors compared to tumors derived from RT3 cells alone or RT3 cells grown with the monolayer fibroblasts. Both the tumor volume and tumor weight were significantly smaller in the fibroblast spheroid group at the end of the experiment (day 34) (Fig. 1(C) and (D)). Similar results were obtained using the NOD/SCID mouse strain (Supplementary figs. S1A and S1B).

3.2. Nemotic fibroblasts enter quiescence

To better understand the tumor growth suppressive function of nemotic fibroblasts we characterized their expression profile using Affymetrix microarrays at 3, 12, 24 and 36 h after initiation of spheroid formation and compared it to that of monolayer fibroblasts. An extensive change in the gene expression pattern, both in number and in fold induction, was seen in the fibroblast spheroids