Experimental Cell Research 336 (2015) 130–140

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Research Article

Endostatin induces proliferation of oral carcinoma cells but its effect on invasion is modified by the tumor microenvironment

Ilkka Alahuhta [a,b], Mari Aikio [c,d], Otto Väyrynen [a,b], Sini Nurmenniemi [a,b], Juho Suojanen [e], Susanna Teppo [a,b], Taina Pihlajaniemi [c,d], Ritva Heljasvaara [c,d], Tuula Salo [a,b,e,f], Pia Nyberg [a,b,*]

[a] Research Group of Cancer and Translational Medicine, Faculty of Medicine, University of Oulu, Finland
[b] Medical Research Center, Oulu University Hospital, Oulu, Finland
[c] Biocenter Oulu and Faculty of Biochemistry and Molecular Medicine, University of Oulu, Finland
[d] Oulu Center for Cell-Matrix Research, University of Oulu, Finland
[e] Department of Oral and Maxillo-facial Diseases, University of Helsinki, Helsinki University Central Hospital, Finland
[f] Department of Oral Diagnosis, School of Dentistry, State University of Campinas, Piracicaba, Sao Paolo, Brazil

ARTICLE INFO

Article history:
Received 20 February 2015
Received in revised form
28 May 2015
Accepted 18 June 2015
Available online 22 June 2015

Keywords:
Endostatin
Extracellular matrix
Organotypic models
Tongue carcinoma
Tumor invasion

ABSTRACT

The turnover of extracellular matrix liberates various cryptic molecules with novel biological activities. Endostatin is an endogenous angiogenesis inhibitor that is derived from the non-collagenous domain of collagen XVIII. Although there are a large number of studies on its anti-tumor effects, the molecular mechanisms are not yet completely understood, and the reasons why endostatin has not been successful in clinical trials are unclear. Research has mostly focused on its anti-angiogenic effect in tumors. Here, we aimed to elucidate how endostatin affects the behavior of aggressive tongue HSC-3 carcinoma cells that were transfected to overproduce endostatin. Endostatin inhibited the invasion of HSC-3 cells in a 3D collagen–fibroblast model. However, it had no effect on invasion in a human myoma organotypic model, which lacks vital fibroblasts. Recombinant endostatin was able to reduce the Transwell migration of normal fibroblasts, but had no effect on carcinoma associated fibroblasts. Surprisingly, endostatin increased the proliferation and decreased the apoptosis of cancer cells in organotypic models. Also subcutaneous tumors overproducing endostatin grew bigger, but showed less local invasion in nude mice xenografts. We conclude that endostatin affects directly to HSC-3 cells increasing their proliferation, but its net effect on cancer invasion seem to depend on the cellular composition and interactions of tumor microenvironment.

1. Introduction

Squamous cell carcinoma (SCC) is the most common type of oral cancer covering over 90% of cancers in the mouth/oral cavity [1]. Despite of new treatments, about 50% of patients die within 5 years [2]. It has been clear about 30 years that the tumor microenvironment (TME) affects tumor growth, invasion and metastasis [3]. The TME consists of several different kinds of cells including fibroblasts, endothelial cells, immune and inflammatory cells as well as extracellular matrix (ECM). ECM consists of *e.g.* collagens, growth factors, proteases and other soluble molecules. Remodeling of the TME during tumor progression can liberate and modify bioactive molecules from the ECM which can affect tumor growth [4]. Some of these cryptic ECM-derived molecules are known to affect cancer progression *via* modulation of tumor angiogenesis.

Endostatin, a 22 kDa non-collagenous fragment of the α1 chain of collagen XVIII [α1(XVIII)NC1], was the first endogenous angiogenesis inhibitor that was discovered as a cryptic fragment of the ECM [5]. In mice it inhibits angiogenesis and tumor growth without any toxicity or side effects [5,6]. Despite the large number

Abbreviations: CAF, Cancer associated fibroblast; ECM, Extracellular matrix; GF, Gingival fibroblast; SCC, Squamous cell carcinoma; TME, Tumor microenvironment; TUNEL, TdT-mediated X-dUTP nick-end-labeling

* Corresponding author at: Research Group of Cancer and Translational Medicine, Faculty of Medicine, University of Oulu, Aapistie 5A, PO Box 5281, FIN-90014 Oulu, Finland.
E-mail address: pia.nyberg@oulu.fi (P. Nyberg).

http://dx.doi.org/10.1016/j.yexcr.2015.06.012
0014-4827/

of studies on the anti-angiogenic and anti-tumor properties of endostatin, its molecular mechanisms are not completely understood. This is largely due to the complexity of the data generated in different experimental conditions. Thus, the effects can depend on the cell type or sub-type [7–10], on the concentration [7,11], on whether endostatin is soluble or immobilized [4,12], or on the presence of certain growth factors [13,14]. In endothelial cells, endostatin affects the expression of up to 12% of all genes including many genes that are not related to angiogenesis [15]. Furthermore endothelial cells are not the only targets, as there is some *in vitro* as well as *in vivo* evidence that endostatin affects other cell types as well, such as prostate cancer cells [7,8,10,16]. However, in most clinical trials endostatin did not improve patient survival, although it is in clinical use in the treatment of lung cancer in China [6] and it has been discovered to be more efficient combined with chemotherapy than chemotherapy alone when threating non-small cell lung cancer [17].

Metastasis, instead of the primary tumor growth, is the main cause for cancer mortality and the TME plays a critical role in the invasion process [18]. It is challenging to develop experimental conditions and methods that would appropriately mimic the real microenvironment of human cancers, but they are crucial in order to reliably elucidate the properties of potential anti-tumor drugs and to find new treatments and to understand the mechanisms of cancer spread. Invasion and migration can be studied either by using cell monolayer assays, Transwell® or other migration assays or more physiological 3D organotypic assays [19]. Our research group has developed a novel human tissue based invasion model utilizing surgically removed myoma tissue [20], which was shown to mimic well the hypoxic TME [21]. In addition, in more than ten studies we have shown that this model is suitable to reliably measure cancer invasion (see *e.g.* [22,23]).

As endostatin has effects on other cell types besides the endothelial cells [7,8,10,24], this study was designed to better understand how it directly affects the behavior of invasive carcinoma cells and the whole TME. To study endostatins effect on cancer invasion, we transfected a highly aggressive human tongue carcinoma cell line HSC-3 to overexpress endostatin and compared it to the HSC-3 cell line transfected with blank vector. Because TME plays such a major role in tumor invasion, we utilized *in vitro* assays as well as mouse tumor burden and various types of organotypic carcinoma invasion models to understand how the endostatin over-expression affects carcinoma cells and the cross-talk with the microenvironment.

2. Materials and methods

2.1. Cell culture

Human tongue squamous cell carcinoma cells HSC-3 (JCRB 0623, Osaka National Institute of Health Sciences, Japan) were cultured in 1:1 DMEM/F-12 media (Invitrogen, Carlsbad, CA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml fungizone, 50 μg/ml ascorbic acid, 0.4 ng/ml hydrocortisone (all from Sigma-Aldrich, Ayrshire, UK) and 10% heat inactivated fetal bovine serum (FBS; Perbio Science, Erembodegem, Belgium). Transfected HSC-3 cell lines were cultured under selective pressure in the presence of 300 μg/ml of Geneticin G418 antibiotic (Invitrogen, Paisley, UK). Primary human gingival fibroblasts (GFs) were obtained from biopsies of healthy gingiva [25]. Cancer associated fibroblasts (CAFs) were derived from tongue squamous cell carcinoma [26]. GFs and CAFs were cultured in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml ascorbic acid, 250 ng/ml fungizone, 1 mM sodium pyruvate (Sigma-Aldrich) and 10% FBS (Perbio Science). All cell lines were used at low passages.

2.2. Endostatin plasmid constructs

For the production of endostatin in mammalian cells, a 550 bp fragment corresponding to the endostatin sequence of human collagen XVIII, preceded by the signal sequence of collagen XVIII long isoform (italics), was generated by PCR using a genomic fragment HP19.3 [27] as a template and the primers 5′-ATTGCCGAATTCATGGCTCCCTACCCCTGTGGCTGCCA-CATCCTGCTGCTGCTCTTCTGCTGCCTGGCGGCTGCCCGGGCCCA-CAGCCACCGCGACTTC-3' and 5′-ATTGCCCTCGAGTCAATGGT-GATGGTGATGATGCTTGGAGGCAGTCATGAAGC-3′(His tag in italics). The amplified PCR fragment was digested with EcoRI and XhoI, cloned to the pcDNA3.1 vector (Invitrogen) and sequenced using ABI PRISM-3100 sequencer. HSC-3 cells were transfected with the endostatin plasmid, or with an empty pcDNA3.1 vector. The transfected cells were selected with 300 μg/ml (HSC-3) or 1000 μg/ml (MDA-MB-435) Geneticin G418 antibiotic (Invitrogen, Paisley, UK) to obtain stable populations of HSC-3 cells expressing human endostatin. The clones used in the experiments were named Ctrl-HSC, Es-HSC(1), Es-HSC(2), Ctrl-MDA, Es-MDA(1) and Es-MDA(2). Detailed transfection protocols have been described earlier [28].

2.3. Organotypic cultures

Two types of organotypic assays were performed; the first assay was based on a mixture of rat tail collagen gel and fibroblasts, and the second assay was based on the use of human myoma tissue. In both of these assays, the HSC-3 cells were cultured on top and they invaded into the underlying tissue or matrix-cell mixture. The collagen gels were prepared as previously described [20]. Briefly, 8 volumes of collagen type I (3.45 mg/ml; BD Biosciences, Bedford, MA), 1 volume of 10 × DMEM (Sigma) and 1 volume of FBS with human gingival fibroblasts (7×10^5 cells) were mixed and allowed to polymerize on 24-well plates at 37 °C for 30 min. After polymerization, 7×10^5 HSC-3 cells (control and endostatin overexpressing cells) were added on top of the gel. Next day the gels were lifted onto collagen-coated (BD Biosciences) nylon disks (Prinsal Oy, Tuusula, Finland) resting on curved steel grids. The grids were placed on 6-well plates and sufficient volume (2.5 ml) of culture media was added to reach the undersurface of the grid generating an air-liquid interface. This was day 1 of the culture.

The myoma organotypic cultures were performed as previously [20]. Briefly, uterine myoma tissue was obtained from routine surgeries after informed consent of the donors (the study was approved by the Ethics Committee of the Oulu University Hospital). The tissue was sliced and myoma disks were made with an 8-mm biopsy punch (Kai Industries Co, Gifu, Japan). The disks were placed into Transwell inserts (diameter 6.5 mm; Corning Incorporated, Corning, NY) and 7×10^5 cancer cells in 50 μl of media were added on top of each myoma disk. The cells were allowed to attach overnight and the myoma disks were transferred onto uncoated nylon disks resting on curved steel grids in 12-well plates with sufficient volume of media (1 ml). The cultured tissues were harvested at day 14 and processed for histology.

2.4. Tumor burden assay in nude mice

The xenograft model was approved by the animal ethics committees of the State Provincial Offices of Oulu and Southern Finland. One million vector control or endostatin overexpressing HSC-3 cells in 200 μl of serum-free media were subcutaneously injected to both flanks of the 11-week old Balb/c nu/nu nude female mice (Harlan, Bicester, UK) ($n=10$ for both groups). Tumor growth was