The International Journal of Biochemistry & Cell Biology 57 (2014) 35–44

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

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

Altered response of fibroblasts from human tympanosclerotic membrane to interacting mast cells: Implication for tissue remodeling

Tadeusz Pawelczyk[a,*], Monika Sakowicz-Burkiewicz[a], Martyna Wesserling[a], Marzena Grden[a], Jerzy Kuczkowski[b]

[a] Department of Molecular Medicine, Poland
[b] Department of Otolaryngology, Medical University of Gdansk, 80-211 Gdansk, Poland

ARTICLE INFO

Article history:
Received 28 April 2014
Received in revised form 5 September 2014
Accepted 2 October 2014
Available online 12 October 2014

Keywords:
Collagens
Fibroblast
Tympanosclerosis
Mast cells
Matrix metalloproteinase-9

ABSTRACT

Several lines of evidence suggest that a tympanosclerotic (TMS) lesion often develops secondary to acute and chronic otitis media. Histological findings indicate that fibroblasts and inflammatory cells, including mast cells, play a key role in the tympanosclerotic plaque formation. However, details on the functional characteristics of tympanosclerotic fibroblasts (Fs^{TMS}) are scanty. Therefore the aim of our study was to examine the activity of human fibroblasts from tympanosclerotic lesions and to evaluate the influence of stimulated by crosslinking of IgE receptor mast cells (HMC-$1^{Fc\varepsilon RI}$) on fibroblast functional behavior. We observed that fibroblasts from normal tympanic membrane (Fs^{TM}) released less TNF-α, TGF-β1 and IL-6 compared to Fs^{TMS}. Fs^{TMS} but not Fs^{TM} upon interaction with HMC-$1^{Fc\varepsilon RI}$ released increased quantities of TNF-α and TGF-β1. Exposing the fibroblast to HMC-$1^{Fc\varepsilon RI}$ cells resulted in an increased synthesis of proteins including collagen. We noted that the *COL2A1* transcript level increased ~5- and ~12-fold in Fs^{TM} and Fs^{TMS} co-cultured with HMC-$1^{Fc\varepsilon RI}$, respectively. Both Fs^{TM} and Fs^{TMS} upon maintenance in the primary culture released significant quantities of matrix metalloproteinase 9 (MMP-9). However, Fs^{TMS} released ~5-fold more MMP-9 activity compared to the Fs^{TM} cultures. The mast cell-induced release of TNF-α, TGF-β1 and MMP-9 sustained for a longer time in Fs^{TMS} cultures compared to Fs^{TM}.

Concluding, our data strongly indicate that increased fibroblast sensitivity to mast cell stimulation greatly contributes to the excessive fibrosis and pathological remodeling of the tympanic membrane. We postulate that the persistency of the Fs^{TMS} activated state could be an important factor in the pathogenesis of tympanosclerosis.

1. Introduction

The term tympanosclerosis indicates pathological changes in the middle ear involving formation of calcified plaques localized in the tympanic membrane and often in the ossicular chain. These calcareous deposits result predominantly in conductive or mixed hearing loss (Asiri et al., 1999). Typically, tympanosclerotic changes proceed *via* the destruction of connective tissue mostly associated with inflammation followed by fibrosis resulting in elevated deposition of collagens, and calcification (Ferlito, 1979). In general, the histology and morphology of tympanosclerotic tissue is well characterized, but the etiology and pathogenesis of tympanosclerosis still remain poorly understood. It is commonly believed that tympanosclerotic lesions develop secondary to acute or chronic inflammation of the middle ear, although other factors such as ventilation tube applications, autoimmune response or genetic predispositions have been reported as etiologies (Moller, 1984; da Costa et al., 1992; Tos and Stangerup, 1989; Schiff and Yoo, 1985; Koc and Uneri, 2002). It should be stressed that there is no known curative treatment, although surgery is commonly performed with mixed results owing to frequently occurring recurrences and risk of iatrogenic sensorineural hearing loss (Tsuzuki et al., 2006; Stankovic, 2009; Aslan et al., 2010).

In the development of tympanosclerosis the involvement of several cytokines, such as IL-6, tumor necrosis factor alpha (TNF-α), transforming growth factor β1 (TGF-β1) and factors like matrix metalloproteinase-9 (MMP-9), osteoprotegerin (OPG), osteopontin (OPN), and nitric oxide have been proposed (Forseni et al., 2001; Makiishi-Shimobayashi et al., 2001; Sziklai et al., 2009; Guo et al., 2012). These compounds are released by the immune cells, as well

* Corresponding author at: Department of Molecular Medicine, Medical University of Gdansk, ul. Debinki 7, paw. 27, 80-211 Gdansk, Poland. Tel.: +48 58 349 2750; fax: +48 58 349 2797.
E-mail address: tkpaw@gumed.edu.pl (T. Pawelczyk).

http://dx.doi.org/10.1016/j.biocel.2014.10.002
1357-2725/

as activated fibroblasts being the major constituent of fibrotic tissue. Mast cells are usually recognized as the tissue-based effector cells in allergic diseases. However, in the last decade accumulated evidence indicates that they are also involved in natural and acquired immunity and Th1-mediated inflammation (Marone et al., 2002; Wooley, 2003). It has been reported that interaction of mast cells and fibroblasts promotes inflammation and tissue remodeling (Garbuzenko et al., 2004; Abel and Vliagoftis, 2008). However, all available data on fibroblasts-driven fibrosis has come from cells that originated from other tissues; mostly heart, liver and lung. Fibroblasts are commonly viewed as uniform cell types having comparable functions regardless of the tissue origination. However, there are reports showing extensive phenotypic differences between fibroblasts from various tissues (Fries et al., 1994; Lekic et al., 1997). The presence of distinct fibroblast subtypes even within the same tissue has been frequently reported (Koumas et al., 2001; Smith et al., 2002). Moreover, recently the reductionist view has been strongly questioned by studies demonstrating that the skin fibroblasts isolated from various anatomical sites displayed a distinct transcriptional pattern, implying that even tissue-specific fibroblast located in different regions of the body should be considered as distinct cell types (Chang et al., 2002). To our best knowledge there is no any information concerning the fibroblast from a tympanic membrane. The involvement of fibroblasts in the development of tympanosclerosis is mainly inferred based on the histological findings indicating increased fibroblast activity and formation of fibrosis. Therefore, in our study we evaluated the activity of fibroblasts from tympanosclerotic lesions and examined the influence of mast cells on fibroblast functional behavior.

2. Materials and methods

2.1. Patients and samples

Tympanic membranes (TM) were obtained from patients with chronic otitis media and tympanosclerosis (TS) who underwent tympanoplasty in the Clinic of Otolaryngology of Medical University of Gdansk. All patients presented no signs of purulent otitis media. Patients with accompanied inflammatory disease, patients with immune deficiency and those taking antibiotics or glucocorticosteroids within the last two weeks were excluded. Morphologically normal tympanic membranes were dissected from persons who had died suddenly. The institutional review board at the Medical University of Gdansk previously approved all procedures (NKEBN/432/2009), and written consent was obtained from all patients. Immediately after resection, tissues were placed in phosphate buffered saline (PBS).

2.2. Cell cultures

Specimens were cut into small pieces, digested for 20 min at 22 °C with 0.25% trypsin made up in PBS and passed through a 100 μm strainer to obtain a single cell suspension. The cells suspension was centrifuged (10 min at 1200 rpm), the pellet was suspended in Dulbecco's modified Eagle's medium (DMEM) containing 20% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), primocin (100 μg/ml) and incubated under standard conditions (5% CO_2–95% air, 98% humidity and 37 °C) for 18 h. Next, all unattached cells were washed out and the remaining cells were cultured in DMEM supplemented with 10% FBS and antibiotics (as above). To obtain a sufficient number of cells for experiments the culture was conducted for 3 weeks. Cells were passaged at ~80% confluence (usually the number of passages ranged from 3 to 4). The fibroblast phenotype of cultured cells was confirmed by light microscopy examination of immunofluorescent labeling with anti-CD34 (clone QBEnd 10), anti-cytokeratin (clone AE1/AE3) and anti-vimentin (clone V9) antibodies (Dako, Glostrup, Denmark). The primary cell cultures were negative for CD 34 and cytokeratin and positive for the mesenchymal marker vimentin (see Supplementary material, Fig. S1).

Human leukemic mast cell line (HMC-1) cells (a kind gift from prof. Michal Wozniak) were maintained under standard conditions in RPMI-1640 supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). To investigate the impact of activated HMC-1 cells on fibroblasts behavior the cells were cocultured at a 1:1 ratio in DMEM for 48 h. In experiments requiring long time culturing the HMC-1 cells were washed out after 48 h and the fibroblast culture was continued.

2.3. HMC-1 cells stimulation

HMC-1 cells normally do not express the high-affinity surface receptor for IgE (FcεRI) (Butterfield et al., 1988). To induce FcεRI on HMC-1 the cells were cultured for 10 days with 20 ng/ml of murine nerve growth factor (NGF) as previously described (Welker et al., 1998). The level of NGF-induced FcεRI expression in HMC-1 cells was evaluated by flow cytometry (see Supplementary material, Fig. S2). HMC-1 cells (possessing FcεRI) were stimulated by cross-linking of the IgE receptor with the purified mouse mAb X-22 (sc-100279, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) directed against the human FcεRIα chain.

2.4. RNA extraction and mRNA level determination

Total RNA was extracted with the use of a Total RNA Prep Plus kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instruction. Before fibroblast harvesting for RNA isolation, the HMC-1 cells (in co-cultures) were washed out with PBS. The efficacy of HMC-1 removing was examined under the light microscope. The gene expression level was determined by real-time PCR performed in a Light Cycler 2.0 (Roche Diagnostic GmbH, Manhein, Germany) using Path-IDTM Multiplex One-Step RT-PCR Kit and appropriate Universal ProbeLibrary Probe for Human (Roche Applied Science). Transcript levels were normalized to that of the β-actin gene (*ACTB*). The primer sequences, TaqMan probes and cycling conditions used are listed in Table 1.

2.5. Measurement of cytokines and growth factors

Cytokines and growth factors were measured in the cell culture media. After sampling the cell culture medium was centrifuged to remove cells and debris. At the time of measurement, the cells in each culture well/dish were counted, and the level of determined factors was normalized to 10^5 cells. The level of tumor necrosis factor alpha (TNF-α) was determined with a Human TNF-alpha US ELISA kit (Invitrogen, Frederick, MD, USA). The level of transforming growth factor β1 (TGF-β1) was assayed using TGF-β1 ELISA kit (Invitrogen, Camarillo, CA, USA). The level of interleukin-6 (IL-6) and interleukin-10 (IL-10) was determined with Interleukin-6 ELISA kit (IBL International GmbH, Hamburg, Germany) and Human IL-10 ELISA kit (Invitrogen, Frederick, MD, USA), respectively. The level of basic fibroblast growth factor (FGF2) was determined with a Human FGF Basic ELISA kit (Invitrogen, Camarillo, CA, USA). The matrix metalloproteinase 9 (MMP-9) was determined with a MMP-9 (human) ELISA kit (Abnova, Jhongli City, Taiwan).

2.6. Determination of collagen and total protein synthesis

Experiments were performed on 3 weeks old primary cultures of fibroblasts. Fibroblasts were plated at a density of 0.25×10^6 cells per well in 6-well tissue culture dishes and allowed to adhere. After