Cytokine 102 (2018) 62–75

Contents lists available at ScienceDirect

Cytokine

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

Molecular and functional characterization of tumor-induced factor (TIF): Hamster homolog of CXCL3 (GRO_γ) displays tumor suppressive activity

Lili Jin[a,1], Zhou-Fang Li[b,1], Da-Kui Wang[b], Meina Sun[a], Wei Qi[b], Qiang Ma[a], Li Zhang[b], Chun Chu[b], Elaine Y.M. Chan[b], Susanna S.T. Lee[c], Helen Wise[b], Ka-Fai To[d], Ying Shi[a], Naiming Zhou[a,*], Wing-Tai Cheung[b,*]

[a] College of Life Sciences, Zhejiang University, Hangzhou, China
[b] School of Biomedical Sciences, The Chinese University of Hong Kong, Hong Kong, China
[c] School of Life Sciences, The Chinese University of Hong Kong, Hong Kong, China
[d] Department of Anatomical and Cellular Pathology, The Chinese University of Hong Kong, Hong Kong, China

ARTICLE INFO

Keywords:
CXCL1
CXCL3
CXCR2
GRO
IL-8
MAS
Tumor-induced factor

Classification:
Chemokine

ABSTRACT

Previously our lab has created a mouse ovarian xenograft model of copy number variation (CNV)-mediated G protein-coupled receptor (GPCR) MAS-driven tumorigenesis, and RNA profiling identified a putative chemokine tumor-induced factor (*Tif*). Sequence analysis and chemotactic study suggested that *Tif* was likely to be a hamster homolog of human *GROγ* (*CXCL3*) [IJC 125 (2009): 1316–1327]. In the present study, we report the molecular and functional characterization of the *Tif* gene. Genomic study of CHO-K1 cells indicated that *Tif* gene consisted of 4 exons, characterized with an antisense *B1* element which is embedded in the fourth exon. Two *Tif* transcripts were identified which shared identical sequences except that a string of 71-nt derived from the antisense *B1* element was deficient in the shorter transcript. Of interests, *B1*-like RNA ladder was detected in xenografts. Functional studies showed that TIF induced chemotaxis and neovessel formation. Pharmacological studies suggested that TIF activated Gi-coupled CXCR2 and induced both calcium mobilization and ERK1/2 phosphorylation, and suppressed forskolin-stimulated cAMP accumulation. In addition, secreted matured TIF functioned as an autocrine factor and promoted anchorage-independent growth. Unexpectedly, TIF delayed the onset of tumor formation, possibly via suppressing proliferation of stromal fibroblasts. However, TIF did not exert any inhibitory effect on tumor growth. Potentially, TIF could be used for preventing cancer relapse.

1. Introduction

Chemokines are a family of small cytokines which mediate leukocyte activation and chemotactic activity. The molecular masses of chemokines range from 8 to 12 kDa. There are four conserved cysteine residues in the primary structure of chemokines, and according to the conserved N-terminal two cysteine residues, the chemokines are divided into four subgroups: CXC, CC, C and CX_3C [1].

The CXC chemokines are usually chemotactic to neutrophils and lymphocytes, and induce the immune cells to migrate from the bloodstream into the surrounding tissue [2]. The CXC chemokines can be further sub-divided into ELR+ and ELR− sub-groups (ELR stands for three amino acid residues: Glu-Leu-Arg). The ELR motif is located at the NH_2-terminus immediately close to the first amino acid residue of the mature peptide, and the presence of the ELR motif suggests that the CXC chemokines are angiogenic [2].

Abbreviations: Ab, antibody; CHO-K1, Chinese hamster ovary-K1; CNV, copy number variation; CXCR2, CXC chemokine receptor 2; CRE, cAMP-response elements; DCIP1, dendritic cell inflammatory protein 1; DIG, digoxigenin; CINC2, cytokine-induced neutrophil chemoattractant 2; DMEM, Dulbecco's modified Eagles's medium; ELISA, enzyme-linked immunosorbent assay; EGFP, enhanced green fluorescent protein; ELR, glutamate-leucine-arginine; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; ERK1/2, extracellular signal-regulated kinase 1/2; F-actin, filamentous actin; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FSK, forskolin; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GRO, growth regulated; HEK293T, human embryonic kidney 293T cell; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ig, immunoglobulins; IL, interleukin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; MEF, mouse embryonic fibroblasts; nt, nucleotide; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMA, 12-O-tetradecanoylphorbol-13-acetate; PKA, protein kinase A; PTX, pertussis toxin; RACE, rapid amplification of 5′-cDNA end; RIPA, radioimmunoprecipitation assay; TBST, Tris-buffered saline and Tween 20; TIF, tumor-induced factor; UTR, untranslated region

* Corresponding authors at: School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China (W.-T. Cheung). College of Life Sciences, Zhejiang University, Zhijinggang Campus, 866 Yuhang Tang Road, Hangzhou 310058, China (N. Zhou).
E-mail addresses: zhounaiming@zju.edu.cn (N. Zhou), wtcheung@cuhk.edu.hk (W.-T. Cheung).
[1] Authors with equal contribution.

https://doi.org/10.1016/j.cyto.2017.12.019
Received 17 April 2017; Received in revised form 11 December 2017; Accepted 15 December 2017

Chemokine Receptors belong to class A GPCR and are sub-divided into different sub-families (CCR, CXCR, CX3CR, XCR, and atypical) according to the chemokines that the receptors bind with. Bindings of chemokines to chemokine receptors are promiscuous; most chemokine receptors recognize more than one chemokines and several chemokines can bind to more than one receptors. Upon activation by chemokines, the receptors trigger G protein-dependent and –independent signaling pathways, such as Gi-mediated adenylate cyclase inhibition, Gi/o-mediated calcium flux and β-arrestin-mediated ERK1/2 phosphorylation [3,4].

GRO CXC chemokines were originally identified from the human malignant melanoma cell line Hs0294 [5], and were subsequently cloned from cDNA libraries of Chinese hamster fibroblastic cells and human bladder tumor cells [6]. Three *GRO* genes (*GROα, β,* and γ; also known as *CXCL1, 2,* and *3*) are identified in human, and homologs of *GRO* are found in rodent as well as other animals. GRO chemokines are potent chemoattractants of neutrophils, mediating inflammation, wound healing and tumorigenesis [7]. All isoforms of GRO chemokines bind mainly to and activate CXCR2 receptors which mediate their cellular effects [3,7]. However, the physiological functions of the three isoforms of GRO chemokines are still unknown.

Abundant expression of chemokines has been reported in inflammatory sites [8] and cancers [9]. Besides acting as autocrine factors to promote tumor growth, GRO chemokines also promote angiogenesis in tumor stroma [10,11]. Angiogenesis is a fundamental step when tumors advance into a malignant state, and the vascularization level of a solid tumor is an excellent indicator of its metastatic potential. GRO-mediated malignant transformation of melanocytes has been shown to involve upregulation of *RAS* expression [12], however, the signaling network that leads to tumor progression is still largely unknown.

Previously, we have identified a novel transcript of ELR+ CXC chemokine and named as tumor-induced factor (*Tif*), in xenografts that were derived from Chinese hamster ovary (CHO) cells overexpressing the GPCR MAS. Sequence analysis and neutrophil migration studies suggested that *Tif* putatively encoded a CXC chemokine of the *GRO* family [13]. In the present study, we report the molecular cloning of the *Tif* gene and the functional characterization of the TIF protein. Furthermore, the potential role of TIF in tumor development was also investigated.

2. Material and methods

2.1. Materials

Cell culture media and fetal bovine serum (FBS) were purchased from HyClone (Logan, USA) or Gibco (Gaithersburg, USA). Opti-MEM® I reduced serum medium and mouse anti-GFP (Cat # 33-2600) were purchased from Invitrogen (Carlsbad, USA). DIG-dUTP, DIG Easy Hyb buffer and FuGENE® HD transfection reagent were purchased from Roche (Mannheim, Germany). TRIzol DNAzol reagent and Lipofectamine 2000 were from Life Technologies (Gaithersburg, USA). The pCMV-Tag4A vector was purchased from Stratagene (La Jolla, USA), pGEM-T vector from Promega (Madison, USA), and pCMV-FLAG and pFLAG-CMV3 vectors were from Sigma (St. Louis, USA). Mouse monoclonal anti-Flag (F3165) was purchase from Sigma (St. Louis, USA) and anti-His_6 (Cat # 27-4710-01) from GE Healthcare (Piscataway, USA). Anti-phospho-ERK1/2 (Thr202/Tyr204) (Cat # 4370) and anti-ERK1/2 (Cat # 4695) antibodies were purchased from Cell Signaling Technology (Danvers, USA), and FITC-phalloidin from Enzo Life Sciences Inc (San Diego, USA). SB225002 was purchased from Cayman Chemicals (Ann Harbor, USA). Forskolin and pertussis toxin (PTX) were purchased from Tocris Bioscience (Ellisville, USA). IL-8 and human GROγ were purchased from PeproTech (Rocky hill, USA). T4 polynucleotide kinase and restriction enzymes were from New England Biolabs (Hitchin, UK). Other chemicals of molecular biology or the highest available grade were purchased from Sigma (St. Louis, USA) or USB (Cleveland, USA).

2.2. Genome structure of TIF (hamster CXCL3)

Genomic DNA of hamster CHO-K1 cells was prepared using DNAzol reagent (Gibco, Gaithersburg, USA) as per manufacturer's instructions. After washing twice with 1.0 mL of 75% ethanol, the DNA pellet was dissolved in 8 mM NaOH and the DNA solution were neutralized with 0.1 M HEPES buffer. *Tif* gene segment was retrieved using polymerase chain reaction (PCR). Briefly, 4 μL (0.1–0.2 μg) of genomic DNA template was added to PCR mixture containing 5.0 μL of 10X PCR buffer, 3.0 μL of $MgCl_2$, 1.0 μL of 10 mM dNTP mix, 2.0 μL each of 10 μM forward primer (5′-GCT CCT GTG CTC CAG ACT T-3′) and 10 μM reverse primer (5′-CCT TTA ATC CCA GCA CTC AG-3′), and sterilized distilled water was added to make up a final volume of 49.5 μL. The PCR mixture was incubated at 94 °C for 5 min, then 0.5 μL of Taq DNA polymerase (5 units/μL) was added immediately and mixed. The PCR reaction was run for 30 cycles with a denature temperature of 94 °C for 30 s, an annealing temperature of 58 °C for 30 s and an extension temperature of 72 °C for 1.5 min. At the end of the amplification cycles, an extending incubation at 72 °C for 7 min was added. The PCR products were cloned into pGEM-T vector (Promega, Madison, USA) for nucleotide sequence determination. Exons and introns were identified by aligning the gene sequences with mRNA sequences. For gene characterization, the sequence was subject to bioinformatics analysis using Web-based servers. The signal peptide of CXCL3 was identified using SignalP server of Technical University of Denmark (http://www.cbs.dtu.dk/services/SignalP), and the 3′-UTR *Alu* sequence was identified by using RepeatMasker Web Server of the Institute for System Biology (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker).

2.3. Cloning of CXCR2 and CXCR1

Full-length cDNAs for human and hamster CXCR2 and CXCR1 were amplified from human and hamster whole genome cDNA library by polymerase chain reaction using gene specific primers (forward, 5′-ccc aag ctt ATG GAA GAT TTT AAC ATG GAG AG-3′ and backward, 5′-cgg ggt acc gaT TAG AGA GTA GTG GAA GTG TGC C-3′ for human CXCR2; forward, 5′-ccc aag ctt ATG GGA GAA ATC AGA GTG GAT AAT-3′ and backward, 5′-cgc gga tcc CAA GGC CAT AAG TAG CCA TGA-3′ for hamster CXCR2; forward, 5′-ccc aag ctt ATG GCC AAG ACT GAA TAT TTC TAC-3′ and backward, 5′-cgc gga tcc tCA AAA AGT AGC ACG ATG ATG TG-3′ for hamster CXCR1). The amplified cDNAs were subcloned into the mammalian cell expression vector, pFLAG-CMV3 (Sigma, St. Louis, USA) and verified by sequencing both strands.

2.4. Generation of TIF-expressing D12 cells

To prepare a stable cell clone overexpressing TIF, full length *Tif* open reading frame (ORF) sequence including the N-terminal signal peptide sequence was PCR amplified with reverse primer containing mutated stop codon (TGA to GCA). A C-terminal tag sequence encoding a thrombin site and a His_6 tag was linked to the 3′-end of the full length *Tif* sequence by overlapping PCR. The resulting PCR amplicons encoding signal peptide-TIF-thrombin site-His_6 fusion peptide was then subcloned into the EcoRI – XhoI sites of pCMV-Tag4A vector (Stratagene, La Jolla, USA) in frame with the Flag tag of the vector, and sequence was confirmed by sequencing. To generate TIF-overexpressing stable clones, CHO-K1 cells (2×10^5 cells/well) were transfected with the pCMV-Tag4A/*Tif* construct using Lipofectamine 2000 (Life technology, Gaithersburg, USA). After transfection and keeping in culture for 24 h, the transfected cells were selected against 1 mg/mL G418. Isolated G418-resistant clones were selected and amplified. Stable transfected colonies derived from single cell were prepared by limiting dilution, and maintained in F12 medium supplemented with 10% FBS and 1 mg/mL G418. Secretion of matured TIF-His_6-Flag peptide into