Bone marrow derived “fibrocytes” contribute to tumor proliferation and fibrosis in gastric cancer

Shiro Terai · Sachio Fushida · Tomoya Tsukada · Jun Kinoshita · Katsunobu Oyama · Koichi Okamoto · Isamu Makino · Hidehiro Tajima · Itasu Ninomiya · Takashi Fujimura · Shinichi Harada · Tetsuo Ohta

Received: 25 February 2014 / Accepted: 10 April 2014 / Published online: 4 May 2014 © The Author(s) 2014. This article is published with open access at Springerlink.com

Abstract

Background Cancer-associated fibroblasts (CAFs) in the stroma are considered to play important roles for gastric cancer proliferation, invasion, and fibrosis, but the source of CAFs and their interaction with cancer cells in the microenvironment have not been fully determined. Here we elucidated the role of bone marrow-derived cells, fibrocytes, in development of gastric cancers, as represented by scirrhous gastric cancer.

Materials and methods In co-culturing MKN45 gastric cancer cells and purified fibrocytes from healthy volunteers, migration and endothelial mesenchymal transition associated gene expression were evaluated using western blot analysis. Also, mouse xenograft models of MKN45 with or without fibrocytes were conducted to investigate their tumorigenicity and immunohistological differences of tumors.

Results Co-culture of fibrocytes with MKN45 resulted in morphological changes from cobblestone-shape to spindle-shape and enhanced expression of α-SMA and collagen type I in fibrocytes, suggesting that co-culture with gastric cancer cells may have induced the differentiation of fibrocytes to myofibroblasts. Furthermore, enhanced expression of SDF-1 in MKN45 and CXCR4 in fibrocytes were also determined. Mouse xenograft models inoculated with MKN45 and fibrocytes revealed significantly larger tumors than those inoculated with MKN45 cells alone, and the stroma in co-inoculated tumors consisted of myofibroblasts and fibrosis. Mouse-derived cells expressing both CD45 and type I collagen were also observed in co-inoculated tumors.

Conclusion Fibrocytes derived from bone marrow may migrate into the microenvironment of gastric cancer by SDF-1/CXCR4 system, and enhance the tumor proliferation and fibrosis as CAFs.

Keywords Fibrocytes · Bone marrow-derived cell · Cancer associated fibroblasts · Cancer stroma · Gastric cancer

Introduction

Tumor tissues are not composed solely of cancer cells, but rather of a mixture of cell types and tissues. These mixtures of various cell types and extracellular matrix are known as the cancer stroma. Cells of the cancer stroma include fibroblasts, endothelial cells that constitute the vascular walls, immune cells such as lymphocytes and macrophages, and other bone marrow-derived cells. The interactions between cancer cells and the adjacent stroma form a complex tumor microenvironment [1–3].

Tumor cells release growth factors that promote tissue fibrosis, including transforming growth factor-β (TGF-β), platelet-derived growth factor, and fibroblast growth factor. These growth factors have been shown to activate fibroblasts in cancer stroma [4].
Fibrocytes contribute to tumor stroma and in fibrotic diseases of the liver, lungs, and kidneys [13–17]. Moreover, they are involved in wound-healing processes and differentiate into fibroblasts and myofibroblasts. Circulating bone-marrow-derived cells that migrate to sites of acute injury and contribute to the process of wound repair [10]. These fibrocytes accounted for 0.1–0.5 % of peripheral blood leukocytes in healthy individuals. Human fibrocytes derived from peripheral blood mononuclear cells can be cultured in plastic dishes, where they appear as adherent, spindle-shaped cells. Furthermore, fibrocytes express surface markers for hematopoietic stem cells (CD34) and a pan-leukocyte marker (CD45), while also producing extracellular matrix molecules including collagen types I and III and fibronectin [11, 12]. Fibrocytes have been reported to migrate into inflammatory tissues and differentiate into fibroblasts and myofibroblasts. Moreover, they are involved in wound-healing processes and in fibrotic diseases of the liver, lungs, and kidneys [13–17].

A high proportion of the tumor mass in patients with scirrhous gastric cancer and peritoneal dissemination is composed of cancer stroma. Although orthotopic fibroblasts may play important roles in the progression, growth, and invasion of gastric cancer [18], the sources of CAFs have not yet been fully determined. Bone marrow-derived cells have been reported to migrate to cancer stroma, where they differentiate into the myofibroblast phenotype [9], but the role of these bone-marrow-derived cells in cancer stroma is unclear. We have therefore assessed whether circulating fibrocytes can differentiate into CAFs and contribute to tumor proliferation and fibrosis.

Materials and methods

Cell lines and cell culture

Peripheral blood was collected from healthy volunteers into conical tubes containing 1–2 mL heparin, and mixed with an equal volume of saline. Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep. A high proportion of the tumor mass in patients with scirrhous gastric cancer and peritoneal dissemination is composed of cancer stroma. Although orthotopic fibroblasts may play important roles in the progression, growth, and invasion of gastric cancer [18], the sources of CAFs have not yet been fully determined. Bone marrow-derived cells have been reported to migrate to cancer stroma, where they differentiate into the myofibroblast phenotype [9], but the role of these bone-marrow-derived cells in cancer stroma is unclear. We have therefore assessed whether circulating fibrocytes can differentiate into CAFs and contribute to tumor proliferation and fibrosis.

Materials and methods

Cell lines and cell culture

Peripheral blood was collected from healthy volunteers into conical tubes containing 1–2 mL heparin, and mixed with an equal volume of saline. Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep.
Similarly, PBMCs were cultured alone or together with an equal number of MKN45 cells for 48 h, and the immunomagnetic cell sorting method was used to separate PBMCs from MKN45 cells. Following trypsinization, the cells were incubated with microbeads coated with anti-human CD326 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany), because CD326 antigen is generally expressed on the surface of cancer cells, but not bone marrow-derived cells. Samples were assayed in triplicate and the results averaged.

Western blotting analysis

Cells were lysed in RIPA buffer (50 mM, pH 8.0, Tris–HCl 150 mM NaCl, 0.5 w/v% sodium deoxycholate, 0.1 w/v% sodium dodecyl sulfate, and 1.0 w/v% NP-40 substitute) (Wako, Tokyo, Japan) containing 1% protease inhibitor cocktail (Sigma-Aldrich). The protein concentration of each sample was measured using a BCA protein assay (Pierce Biotechnology, Rockford, IL, USA). Whole-cell lysates were prepared in denaturing SDS sample buffer, subjected to SDS-PAGE (ATTO Co. Ltd., Tokyo, Japan), and transferred to nitrocellulose membranes. These membranes were incubated with primary antibodies to CD34 (H-140, rabbit polyclonal IgG, diluted 1:1,000; Santa Cruz Biochemistry), αSMA (1A4, mouse monoclonal IgG, diluted 1:100; Santa Cruz Biochemistry), E-cadherin (H-108, rabbit monoclonal IgG, diluted 1:5,000; DakoCytoMation, Copenhagen, Denmark), and β-actin (AC-15, mouse monoclonal IgG, diluted 1:10,000; Sigma-Aldrich). Antibody binding was visualized using ECL Western-Blotting detection kits (GE Healthcare, Waukesha, WI, USA) and the Light-Capture system (ATTO), and then quantified using the CS analyzer program (ATTO). All experiments were repeated three times.

Mouse xenograft model

All animal experiments conformed to the guidelines of the Kanazawa University for the care and use of laboratory animals. BALB/c nu/nu female mice aged 4–5 weeks were obtained from Charles River Laboratories Inc. (Japan). Fibrocytes were stained with red fluorescent dye PKH26 (4 μM) using a cell linker kit (Sigma-Aldrich) according to the manufacturer’s instruction. Each of the eight control mice was inoculated $7 \times 10^6$ of MKN45 cells in 100 μL of RPMI-1640 subcutaneously, whereas each of the eight experimental mice was inoculated $5 \times 10^6$ of MKN45 cells plus $2 \times 10^6$ of fibrocytes in 100 μL of RPMI-1640. Xenograft tumors were measured every day for 11 days. Tumor volume was estimated using the equation $v = (ab^2)/2$, in which $v$ is volume, $a$ is the length of the major axis, and $b$ is the length of the minor axis. After 10 days, the mice were sacrificed, and the tumors were removed for immunohistochemical examination.

Histological and immunohistochemical examination

Subcutaneous tumor specimens removed from xenografted mice were shock frozen in liquid nitrogen, cryosectioned, mounted onto glass slides, and air dried. The samples were analyzed by fluorescence microscopy using a standard filter setup for visualization of PKH26. Other samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin or Mallory–Azan stain and observed under a light microscope. Other tissue samples were immunostained with antibody to αSMA (1A4, mouse monoclonal IgG, diluted 1:100; DakoCytoMation) overnight at 4 °C. The sections were treated with EnVision reagent (Dako Co., Japan) for visualization. In addition, sections were doubly immunostained with specific antibodies against CD45 and collagen type I to assess the numbers of mouse-derived cells by dual immunohistochemical techniques. The slides were immersed in methanol containing 0.3% H$_2$O$_2$ for 30 min, blocked with 3.3% normal goat serum in PBS and incubated with rat anti-mouse CD45 polyclonal antibodies (1:100; R&D Systems, Minneapolis, MN, USA) and rabbit anti-mouse collagen type I (1:100; Santa Cruz Biotechnology) for 2 h at room temperature. After washing in PBS, the sections were incubated with anti-rat IgG antibody conjugated with Alexa Fluor® 488 and anti-rabbit IgG antibody conjugated with Alexa Fluor® 546 (Molecular Probes/Life Technologies) (1:400) for 1 h at room temperature. Nuclei were counterstained with DAPI (diluted 1:1,000; Molecular Probes/Life Technologies) for 5 min and the slides viewed with an immunofluorescence microscope (BX50/BX-FLA; Olympus, Japan).

Statistical analysis

Results are expressed as means ± standard deviations and compared using one-way analysis of variance or two-sided Student’s $t$ tests. All statistical analyses were performed using the computer software package SPSS 10.0 (SPSS Inc., USA), with $p < 0.05$ indicating a statistically significant difference.

Results

TGF-β induced differentiation of PBMCs to fibrocytes and myofibroblasts

Peripheral blood mononuclear cells contain lymphocytes (B cells and T cells), monocytes, and fibrocytes precursors.
Using immunomagnetic cell sorting, we found that the purity of fibrocytes originating from cultured PBMCs was almost 90% (Fig. 1).

Adherent cells from PBMC preparations gradually elongated, showing a spindle-shaped morphology with an oval nucleus. The addition of TGF-β1 (5 ng/mL) to PBMC cultures on day 7 promoted fibrocytes differentiation, as shown by the increased percentage of cultured cells that were spindle shaped (Fig. 2a, b). In addition, western blotting analysis showed an increase in collagen type I and α-SMA expression, along with a decrease in CD34 expression (Fig. 2c). These results suggested that TGF-β1 promoted the differentiation of PBMCs into fibrocytes and myofibroblasts.

Effect of co-culturing with gastric cancer cells and fibrocytes

Western blot analysis showed that indirect co-culturing of MKN45 cells with fibrocytes increased expression of SDF-1 and decreased expression of E-cadherin (Fig. 3a). In contrast, fibrocytes directly co-cultured with MKN45 cells and separated by immunomagnetic cell sorting showed increased expression of collagen type I, αSMA, and CXCR4, and decreased expression of CD34 (Fig. 3b).
Human gastric cancer cells co-cultured with fibrocytes significantly formed large mouse xenograft tumors

The size of tumors from inoculated MKN45 cells plus fibrocytes was larger than that of MKN45 cells alone (Fig. 4), with the difference being statistically significant 7 days after inoculation ($p < 0.05$). The former tumors also had larger areas of fibrosis (Fig. 5a, b) and enhanced α-SMA expression (Fig. 5c). Moreover, a higher percentage of exogenous fibrocytes in co-inoculated tumors were positive for α-SMA expression (Fig. 5d). To determine whether endogenous host fibrocytes migrated into these tumors, the tumors were stained with antibodies to mouse COLA1A and CD45, antibodies that did not cross-react with human proteins. The co-inoculated tumors contained cells positive for both antigens, strongly indicating that mouse fibrocytes also participated in tumor formation (Fig. 6a–d).

Discussion

We have shown that bone-marrow-derived circulating fibrocytes differentiated into myofibroblasts and contributed to tumor proliferation and fibrosis in a mouse xenograft model of gastric cancer. We also found that these tumors contained cells with the phenotypic characteristics of mouse bone marrow.

Cancer stroma is closely associated with the promotion of tumor growth and acquisition of invasive capacity. CAFs in tumors and surrounding tissues were recently reported to play important roles in the formation of tumor microenvironments [2, 7], including in gastric cancer. The percentage of orthotopic fibroblasts co-cultured with gastric cancer cells that differentiated into myofibroblasts was significantly higher than the percentage of normal orthotopic fibroblasts [19]. In addition, myofibroblasts in the
microenvironment may stimulate gastric cancer cell motility, including migration and invasion [20]. Although the origins of CAFs have not yet been fully defined, they are thought to arise from multiple sites, such as neighboring tissues and remote organs. In addition, several studies have shown that bone marrow-derived cells are involved in the formation of cancer stroma [9, 21].

Peripheral blood circulating fibrocytes migrate to inflamed tissue sites in response to chemokines released by inflammatory cells [10], where they differentiate into fibroblasts and myofibroblasts and contribute to tissue inflammation and fibrosis formation [14, 22]. We found that co-culturing of fibrocytes with gastric cancer cells induced a fibroblast-like morphology in the former, and
up-regulated their expression of αSMA and type I collagen, findings similar to those observed when PBMCs were cultured in the presence of TGF-β. Cancer cell-derived TGF-β has been reported to modulate myofibroblast differentiation in colon and breast cancer [23, 24], suggesting that cancer-cell-derived TGF-β may also induce differentiation of fibrocytes into myofibroblasts.

MKN45 cells were derived from hepatic metastatic tumor with a microscopic phenotype that was a solid type of poorly differentiated adenocarcinoma (por1). In this study, even MKN45 could acquire the ability of transforming fibrocytes. Most of gastric cancer cell lines were derived from various tumors that consist of cancer cells and stroma. Except for latent ability of stromal induction, MKN45 cells were considered to be appropriate in this study.

Subcutaneous co-implantation of MKN45 cells and fibrocytes formed significantly larger tumors and significantly greater stromal proliferation than implantation of MKN45 cells alone. The stroma in the co-implanted tumors contained collagen fibers and αSMA-expressing myofibroblasts, as well as a higher proportion of PKH26-positive cells. These findings indicated that bone marrow-derived fibrocytes may have contributed to the proliferation of xenograft tumors and differentiated toward myofibroblasts.

In addition, by using a subcutaneous model, it was possible to distinguish the migrated cells from orthotopic fibroblasts by mouse-specific antibodies. Immunohistochemical staining with antibodies against mouse CD45 and collagen type I antibodies (red). Lower left Nuclear staining (blue). Lower right Merged images.

Fig. 6 Dual immunohistochemical staining of mouse xenograft tumors with specific antibodies against CD45 and collagen type I, as shown by immunofluorescent microscopy (×400). Each colored arrow indicates a cell that is both mouse specific CD45 antigen positive and collagen type I positive. Upper left Immunostaining with rat anti-mouse CD45 antibodies (green). Upper right Immunostaining with rabbit anti-mouse collagen type I antibodies (red). Lower left Nuclear staining (blue). Lower right Merged images.

Various cells other than CAFs contribute to the tumor microenvironment. For example, we have shown that activated peritoneal mesothelial cells co-cultured with MKN45 cells may promote tumor growth and fibrosis [26].

In conclusion, bone marrow-derived cells might migrate in a microenvironment of gastric cancer and differentiate into myofibroblasts. These myofibroblasts subsequently contribute to tumor formation and fibrosis as CAFs.
Acknowledgments This study was supported in part by Grants-in-Aid for Scientific Research (C; 22591451 to S.F.) from the Japan Society for the Promotion of Science (JSPS).

Conflict of interest None of the authors have conflicts of interest to decline.

Open Access This article is distributed under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and the source are credited.

References

1. Tisty TD. Stromal cells can contribute oncogenic signals. Semin Cancer Biol. 2001;11:97–104.
2. Mueller MM, Fusenig NE. Friends or foes—bipolar effects of the tumour stroma in cancer. Nat Rev Cancer. 2004;4:839–49.
3. Schäfer M, Werner S. Cancer as an overhealing wound: an old hypothesis revisited. Nat Rev Mol Cell Biol. 2008;9:628–38.
4. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002;420:860–7.
5. Wever OD, Demetter P, Mareel M, Bracke M. Stromal myofibroblasts are drivers of invasive cancer growth. Int J Cancer. 2008;123:2229–38.
6. Semba S, Kodama Y, Ohmura K, Mizuuchi E, Masuda R, Yashiro M, et al. Direct cancer-stromal interaction increases fibroblast proliferation and enhances invasive properties of scirrhous-type gastric carcinoma cells. Br J Cancer. 2009;101:1365–73.
7. Kalluri R, Zeisberg M. Fibroblasts in cancer. Nat Rev Cancer. 2006;6:392–401.
8. Shimoda M, Melody KT, Orimo A. Carcinoma-associated fibroblasts are a rate-limiting determinant for tumour progression. Semin Cell Dev Biol. 2010;21:19–25.
9. Direkze NC, Hodivala-Dilke K, Jeffery R, Hunt T, Poulsom R, Oukrif D, et al. Bone marrow contribution to tumor-associated myofibroblasts and fibroblasts. Cancer Res. 2004;64:8492–5.
10. Bucala R, Spiegel LA, Chesney J, Hogan M, Cerami A. Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. Mol Med. 1994;1:71–81.
11. Pilling D, Fan T, Huang D, Kaul B, Gomer RH. Identification of markers that distinguish monocyte-derived fibrocytes from monocytes, macrophages, and fibroblasts. PLoS One. 2009;4(4):e5475.
12. Bellini A, Mattoli S. The role of the fibrocyte, a bone marrow-derived mesenchymal progenitor, in reactive and reparative fibroses. Lab Invest. 2007;87:858–70.
13. Herzog EL, Bucala R. Fibrocytes in health and disease. Exp Hematol. 2010;38:548–56.
14. Abe R, Donnelly SC, Peng T, Bucala R, Metz CN. Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. J Immunol. 2001;166:7556–62.
15. Moeller A, Gilpin SE, Ask K, Cox G, Cook D, Gauldie J, et al. Circulating fibrocytes are an indicator of poor prognosis in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med. 2009;179:588–94.
16. Sakai N, Furuiuchi K, Shinozaki Y, Yamauchi H, Toyama T, Kitajima S, et al. Fibrocytes are involved in the pathogenesis of human chronic kidney disease. Hum Pathol. 2010;41:672–8.
17. Phillips RJ, Burdick MD, Hong K, Lutz MA, Murray LA, Xue YY, et al. Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. J Clin Invest. 2004;114:438–46.
18. Fuyuhiro Y, Yashiro M, Noda S, Matsuoka J, Hasegawa T, Kato Y, et al. Cancer-associated orthotopic myofibroblasts stimulates the motility of gastric carcinoma cells. Cancer Sci. 2012;103:797–805.
19. Fuyuhiro Y, Yashiro M, Noda S, Kashiwagi S, Matsuoka J, Doi Y. Upregulation of cancer-associated myofibroblasts by TGF-β from scirrhous gastric carcinoma cells. Br J Cancer. 2011;105:996–1001.
20. Zhi K, Shen X, Zhang H, Bi J. Cancer-associated fibroblasts are positively correlated with metastatic potential of human gastric cancers. J Exp Clin Cancer Res. 2010;29:66.
21. Ishii G, Sangai T, Ito T, Hasebe T, Endoh Y, Sasaki H, et al. In vivo and in vitro characterization of human fibroblasts recruited selectively into human cancer stroma. Int J Cancer. 2005;117:212–20.
22. Hong KM, Belperio JA, Keane MP, Burdick MD, Strieter RM. Differentiation of human circulating fibrocytes as mediated by transforming growth factor-β and peroxisome proliferator-activated receptor γ. J Biol Chem. 2007;282:22910–20.
23. Denys H, Derycke L, Hendrix A, Wesbrooke W, Gheldof A, Narine K, et al. Differential impact of TGF-β and EGF on fibroblast differentiation and invasion reciprocally promotes colon cancer cell invasion. Cancer Lett. 2008;266:263–74.
24. Koijima Y, Acar A, Eaton EN, Melody KT, Scheel C, Ben-Porath I, et al. Autocrine TGF-β and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts. Proc Natl Acad Sci USA. 2010;107:20009–14.
25. Mehrad B, Burdick MD, Strieter RM. Fibrocyte CXCR4 regulation as a therapeutic target in pulmonary fibrosis. Int J Biochem Cell Biol. 2009;41:1708–18.
26. Tsukada T, Fushida S, Harada S, Yagi Y, Kinoshita J, Oyama K, et al. The role of human peritoneal mesothelial cells in the fibrosis and progression of gastric cancer. Int J Oncol. 2012;41:476–82.