Tumor-Associated Macrophage-Induced Invasion and Angiogenesis of Human Basal Cell Carcinoma Cells by Cyclooxygenase-2 Induction

Jeng-Wei Tjiu[1], Jau-Shiuh Chen[1], Chia-Tung Shun[2,3], Sung-Jan Lin[1], Yi-Hua Liao[1], Chia-Yu Chu[1], Tsen-Fang Tsai[1], Hsien-Ching Chiu[1], Yang-Shia Dai[1], Hiroyasu Inoue[4], Pan-Chyr Yang[5], Min-Liang Kuo[6,7] and Shiou-Hwa Jee[1,7]

Tumor-associated macrophages (TAMs) and cyclooxygenase-2 (COX-2) are associated with invasion, angiogenesis, and poor prognosis in many human cancers. However, the role of TAMs in human basal cell carcinoma (BCC) remains elusive. We found that the number of TAMs infiltrating the tumor is correlated with the depth of invasion, microvessel density, and COX-2 expression in human BCC cells. TAMs also aggregate near COX-2 expressing BCC tumor nests. We hypothesize that TAMs might activate COX-2 in BCC cells and subsequently increase their invasion and angiogenesis. TAMs are a kind of M2 macrophage derived from macrophages exposed to Th2 cytokines. M2-polarized macrophages derived from peripheral blood monocytes were cocultured with BCC cells without direct contact. Coculture with the M2 macrophages induced COX-2-dependent invasion and angiogenesis of BCC cells. Human THP-1 cell line cells, after treated with phorbol myristate acetate (PMA), differentiated to macrophages with M2 functional profiles. Coculture with PMA-treated THP-1 macrophages induced COX-2-dependent release of matrix metalloproteinase-9 and subsequent increased invasion of BCC cells. Macrophages also induced COX-2-dependent secretion of basic fibroblast growth factor and vascular endothelial growth factor-A, and increased angiogenesis in BCC cells.

Journal of Investigative Dermatology (2009) **129,** 1016–1025; doi:10.1038/jid.2008.310; published online 9 October 2008

INTRODUCTION

Basal cell carcinoma (BCC) is the most common human cancer; nearly one million new cases occur in the United States every year (Miller and Weinstock, 1994). BCC usually grows slowly and rarely metastasizes. If left untreated, BCC may invade deep layers of the skin and cause local tissue destruction (Walling *et al.*, 2004). The progression of human BCC is highly influenced by the host immune response and the inflammatory cells in the tumor microenvironment (Kaur *et al.*, 2006; Kaporis *et al.*, 2007). Among the host inflammatory cells, macrophages play a pivotal role.

Macrophages have functional plasticity and can change their functional profiles repeatedly in response to environmental changes (Watkins *et al.*, 2007). Like the Th1/Th2 nomenclature, the two extremes of polarized macrophages are named M1 and M2 macrophages (Martinez *et al.*, 2006). When macrophages are exposed to lipopolysaccharides (LPS) and IFN-γ, they are polarized to M1 macrophages and have antitumor activities. When they are exposed to Th2 cytokines, such as IL-4 and IL-13, they are polarized to M2 macrophages and support tumor growth (Mantovani *et al.*, 2002). M2 macrophages, compared with M1 macrophages, produce low amounts of tumor necrosis factor-α (TNF-α), IL-1β, IL-6, and high amounts of transforming growth factor-β (TGF-β). M2 macrophages also express surface markers such as CD206 (mannose receptor) and CD204 (scavenger receptor A) (Mantovani *et al.*, 2004).

Tumor-associated macrophages (TAMs), referring to the macrophages residing in the tumor microenvironment, are a kind of M2 macrophage (Sica *et al.*, 2006). TAMs can promote tumor growth, invasion, metastasis, and activate

[1]*Department of Dermatology, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan;* [2]*Department of Pathology, National Taiwan University Hospital, Taipei, Taiwan;* [3]*Department of Forensic Medicine, National Taiwan University College of Medicine, Taipei, Taiwan;* [4]*Department of Food Science and Nutrition, Nara Women's University, Nara, Japan;* [5]*Department of Internal Medicine, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan and* [6]*Laboratory of Molecular and Cellular Toxicology, Graduate Institute of Toxicology, National Taiwan University College of Medicine, Taipei, Taiwan*

[7]*These authors contributed equally to this work.*

Correspondence: Dr Shiou-Hwa Jee and Dr Min-Liang Kuo, Department of Dermatology, National Taiwan University Hospital and National Taiwan University College of Medicine, 7 Chung-Shan South Road, Taipei, Taiwan. E-mails: shiouhwa@ntu.edu.tw or shiouhwa@gmail.com

Abbreviations: BCC, basal cell carcinoma; bFGF, basic fibroblast growth factor; COX-2, cyclooxygenase-2; FCS, fetal calf serum; HUVEC, human umbilical vein endothelial cell; LPS, lipopolysaccharides; MMP, matrix metalloproteinase; MVD, microvessel density; NF-κB, nuclear factor-κB; PMA, phorbol myristate acetate; TAM, tumor-associated macrophages; TGF, transforming growth factor-β; TNF-α, tumor necrosis factor; VEGF-A, vascular endothelial growth factor-A

Received 14 February 2008; revised 24 July 2008; accepted 21 August 2008; published online 9 October 2008

tumor-promoting genes in cancer cells (Chen *et al.*, 2003; Lewis and Pollard, 2006). Previous reports showed that after lung cancer cells were cocultured with macrophages in a noncontact system, cyclooxygenase-2 (COX-2) expression in lung cancer cells was significantly induced (Chen *et al.*, 2005). Our previous paper showed that overexpression of COX-2 in BCC cells promoted tumorigenesis and angiogenesis (Tjiu *et al.*, 2006). However, the role of TAMs in human BCC was not clear. In this report, we show that the number of TAMs correlated with the depth of invasion and microvessel counts in human BCC. In addition, we also found that TAMs aggregated near COX-2-expressing cancer nests in human BCC. Similar spatial adjacencies were reported in prostate cancer and hepatocellular carcinoma (Cervello *et al.*, 2005; Wang *et al.*, 2005). We hypothesize that TAMs might activate COX-2 in BCC cells and subsequently increase their invasion and angiogenesis.

We used M2-polarized human THP-1 macrophages and monocyte-derived M2 macrophages to coculture with BCC cells without direct contact in a Transwell apparatus. Our results show that M2 macrophages induced BCC cell invasion and angiogenesis in a COX-2-dependent manner.

RESULTS

Higher numbers of TAMs correlated with an increased depth of invasion, angiogenesis, and COX-2 expression in BCC cells

Immunohistochemistry analysis showed conspicuous TAM aggregations close to COX-2-postitive tumor nests. TAMs also colocalized with prominent neovascularization in an aggressive (micronodular) subtype of BCC (Figure 1). A higher number of TAMs was associated with a clear trend of increasing depth of invasion and a higher microvessel density (MVD; both $P<0.01$; Table 1). COX-2 expression in epithelial cells of human BCC was assayed semiquantitatively and scored as: grade 0, <10%; grade 1, 10–50%; or grade 2 >50% for epithelial cells stained positive for COX-2. A higher number of TAMs also correlated with a higher grade of COX-2 expression in epithelial cells of human BCC ($P=0.0001$, Kruskal–Wallis test; Table 1). To adjust for the effects of other clinicopathological factors on the depth of invasion and MVD, we used a multivariate linear regression model. Both the number of TAMs and COX-2 expression in epithelial cells remained significant predictors for invasion and angiogenesis (Table 2). If the number of TAMs increases by 1, the predicted depth of invasion will increase by 0.01 mm and the predicted MVD will increase by 0.17/high-power field. If the grade of COX-2 expression increases by 1, the predicted depth of invasion will increase by 0.62 mm and the MVD will increase by 13.91/high-power fields. These data suggested an interaction between TAMs and COX-2 expression in BCC cancer cells, with both being important for BCC invasion and angiogenesis.

PMA treatment differentiated THP-1 cells to M2 macrophages

Human THP-1 cells are widely used as models for monocyte/macrophages differentiation. When treated with phorbol myristate acetate (PMA, 320 nM) for 24 hours, THP-1 cells quickly stopped proliferating, became attached, differentiated

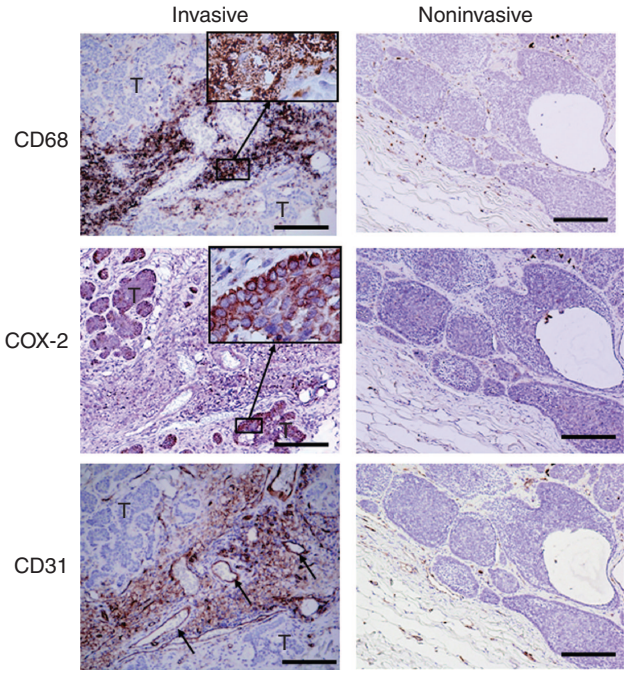

Figure 1. High number of TAMs located near COX-2-expressing epithelial cell nests and increased microvessel in BCC. Serial sections showed that aggressive (micronodular) subtype BCC had increased number of CD68-positive macrophages aggregated adjacent to COX-2-expressing epithelial cancer nests. Increased number of CD31-positive microvessels located in area of dense macrophages infiltration. (× 100; inset × 400). Bars = 20 μm.

to monocytes (CD14 positive), and subsequently to macrophages (CD68 positive; Figure 2a). The PMA-treated THP-1 macrophages exhibited significant expression of M2 macrophage surface markers CD206 (mannose receptor) and CD204 (scavenger receptor A; Figure 2a).

Macrophages had a high degree of functional plasticity as they could change their functional profiles (from M1 to M2 or M2 to M1) repeatedly depending on the cytokines (Th1 or Th2) to which they were exposed to (Watkins *et al.*, 2007). If M2 macrophages were exposed to Th1 cytokines, they were polarized to M1; when M1-polarized macrophages were exposed to Th2 cytokines, they were polarized to M2. We hypothesized that PMA-treated THP-1 macrophages were M2 macrophages. If PMA-treated THP-1 macrophages were exposed to Th1 cytokines (LPS and IFN-γ), they were polarized to M1 macrophages and would show a M1 cytokine profile. When PMA-treated THP-1 macrophages were further treated with Th2 cytokines (IL-4/IL-13), they would remain M2 polarized and have a M2 cytokine profile. On the basis of this, we generated M1-polarized THP-1 macrophages by treating THP-1 cells with PMA for 24 hours and polarizing them with Th1 cytokines (20 ng ml^{-1} IFN-γ and 100 ng ml^{-1} LPS) for 18 hours (added 6 hours after PMA). M2-polarized THP-1 macrophages were obtained from THP-1 cells treated with PMA for 24 hours with 18-hours treatment of Th2 cytokines (20 ng ml^{-1} IL-4 and 20 ng ml^{-1} IL-13, added 6 hours after PMA; schema shown in Figure 2b). The cytokine profiles of the PMA-treated, M1-polarized, and M2-polarized THP-1

Table 1. Increased numbers of TAMs are associated with increased depth of invasion, microvessel density, and epithelial COX-2 expression in human BCC

		Depth of invasion (mm)		MVD (no./HPF)[1]		BCC cells COX-2 expression[2,3]		
Number of TAM/HPF[1]	*n*	Mean ± SD	*P*[4]	Mean ± SD	*P*[4]	Grade 0	Grade 1	Grade 2
<117	30	1.41 ± 0.95	<0.01*	31.8 ± 14.1	<0.01*	7	20	3
117–215	31	1.76 ± 0.96		44.9 ± 21.3		3	16	12
>215	31	3.32 ± 1.57		85.6 ± 21.5		0	2	29
Total	92	2.17 ± 1.45		54.4 ± 29.9		10	38	44

MVD, microvessel density; TAMs, tumor-associated macrophages; COX-2, cyclooxygenase-2; HPF, high-power field; SD, standard deviation.
[1]Number of TAMs in ×200HPF was divided into tertiles.
[2]COX-2 expression in human BCC cells was assayed semiquantitatively and scored as: grade 0, <10%; grade 1, 10–50%; grade 2 >50% for BCC cells that stained positive for COX-2.
[3]Kruskal–Wallis test; P=0.0001.
[4]Test for trend of ordered categories; $P<0.05$ (*) was considered significant.

Table 2. Multivariate linear regression analysis identified the number of TAMs and the grade of COX-2 expression as a significant predictor for depth of invasion and MVD in human BCC

	Invasion depth (mm)			MVD (no./ HPF)		
Characteristics[1]	Coefficient	SE	*P*-value[2]	Coefficient	SE	*P*-value[2]
Age (years)	0.00	0.01	0.61	−0.06	0.12	0.59
Sex (male/female)	0.05	0.21	0.82	3.08	3.21	0.34
Head and neck location vs non-head and neck location	0.48	0.27	0.09	0.89	4.19	0.83
Aggressive vs nonaggressive histological subtypes	−0.19	0.23	0.42	7.98	3.49	0.03*
Grade of BCC cells COX-2 expression	0.62	0.21	<0.01*	13.91	3.28	<0.01*
Number of TAM/ × 200 HPF	0.01	0.00	<0.01*	0.17	0.02	<0.01*
Tumor diameter	0.13	0.24	0.58	1.67	3.69	0.65
Recurrent vs primary BCC	0.03	0.51	0.96	6.86	7.77	0.38
With vs without history of skin cancer (other than BCC)	−0.71	0.45	0.12	2.77	6.34	0.69
With vs without history of radiotherapy	1.19	0.68	0.08	−8.99	10.43	0.39

TAMs, tumor-associated macrophages; COX-2, cyclooxygenase-2; MVD, microvessel density; BCC, basal cell carcinoma; HPF, high-power field; SE, standard error.
[1]Age, macrophage density, and tumor diameter (mm) were continuous variables. Head and neck location, aggressive histological subtype, recurrent BCC, history of skin cancer (other than BCC), and history of radiotherapy were ordinal variables. Grade of COX-2 expression is categorical.
[2]$P<0.05$ (*) is regarded as significant.

macrophages were compared. The PMA-treated and M2-polarized THP-1 macrophages shared the same profile, that is low TNF-α, IL-1β, IL-6, and high TGF-β, which was exact opposite to that of M1-polarized THP-1 macrophages (Figure 2b). Taking the cytokine profiles and surface markers together, PMA treatment induced a M2 response in THP-1 macrophages.

M2-polarized THP-1 macrophages induced COX-2-dependent invasion and angiogenesis of BCC cells

We then used PMA-treated and M2-polarized THP-1 macrophages to coculture with BCC cells in a noncontact system. The protocol is shown in Figure S1. For PMA-treated THP-1 cells, 1×10^6 THP-1 cells were seeded into upper insert of a six-well Transwell and treated with 320 nM PMA for 24 hours (Figure 2c). For M2-polarized THP-1 macrophages, 320 nM PMA was added to THP-1 cells for 6 hours, followed by PMA plus 20 ng ml^{-1} of IL-4/IL-13 in the following 18 hours (Figure 2c). PMA-containing media were all removed and cells washed three times in PBS to remove all PMA. PMA-treated or M2-polarized THP-1 macrophages (in upper inserts) were then cocultured with BCC cells (in six-well plate) for 6 hours. After coculture, macrophages (upper inserts) were discarded, and BCC cells were assayed for invasion and *in vitro* angiogenesis.

For invasion assay, BCC cells (in six-well plate) were seeded into Matrigel-coated invasion chambers (24 wells, 8 μm pore size). After 24 hours, BCC cells were fixed, stained with crystal violet, and counted for invaded cells. BCC cells that had been cocultured with PMA-treated or M2-polarized THP-1 macrophages showed an increased number of invaded cells (both $P<0.05$; Figure 2d). However, when BCC cells

were transiently transfected with COX-2 siRNA or pretreated with celecoxib (10 μM) for 30 minutes before they cocultured with macrophages, the increments were abrogated (all $P<0.05$; Figure 2d).

For the *in vitro* angiogenesis assay, BCC cells that had been cocultured with macrophages were washed and serum-free RPMI 1640 were added. At 24 hours later, BCC culture supernatants (conditioned media) were collected. Concentrated conditioned media (10-fold) were added to human umbilical vein endothelial cells (HUVECs). The numbers of tube-like structures formed by HUVECs were determined 6 hours later. The supernatants derived from BCC cells that had been cocultured with PMA-treated or M2-polarized THP-1 macrophages showed increased numbers of tube-like structures of HUVECs (both $P<0.05$; Figure 2e). The increments were blocked by transiently transfecting BCC cells with COX-2 siRNA or treating BCC cells with celecoxib (10 μM) 30 minutes before coculture (all $P<0.05$; Figure 2e). These results suggested M2 macrophages induced a COX-2-dependent invasion and angiogenesis in human BCC cells.

TAMs activate p38 MAPK/NF-κB/COX-2 cascades and induce MMP-9/VEGF-A/bFGF expression

Significant induction of COX-2 mRNA was found in BCC cells that had been cocultured with PMA-treated or M2-polarized THP-1 macrophages (both $P<0.05$; Figure 3a). To

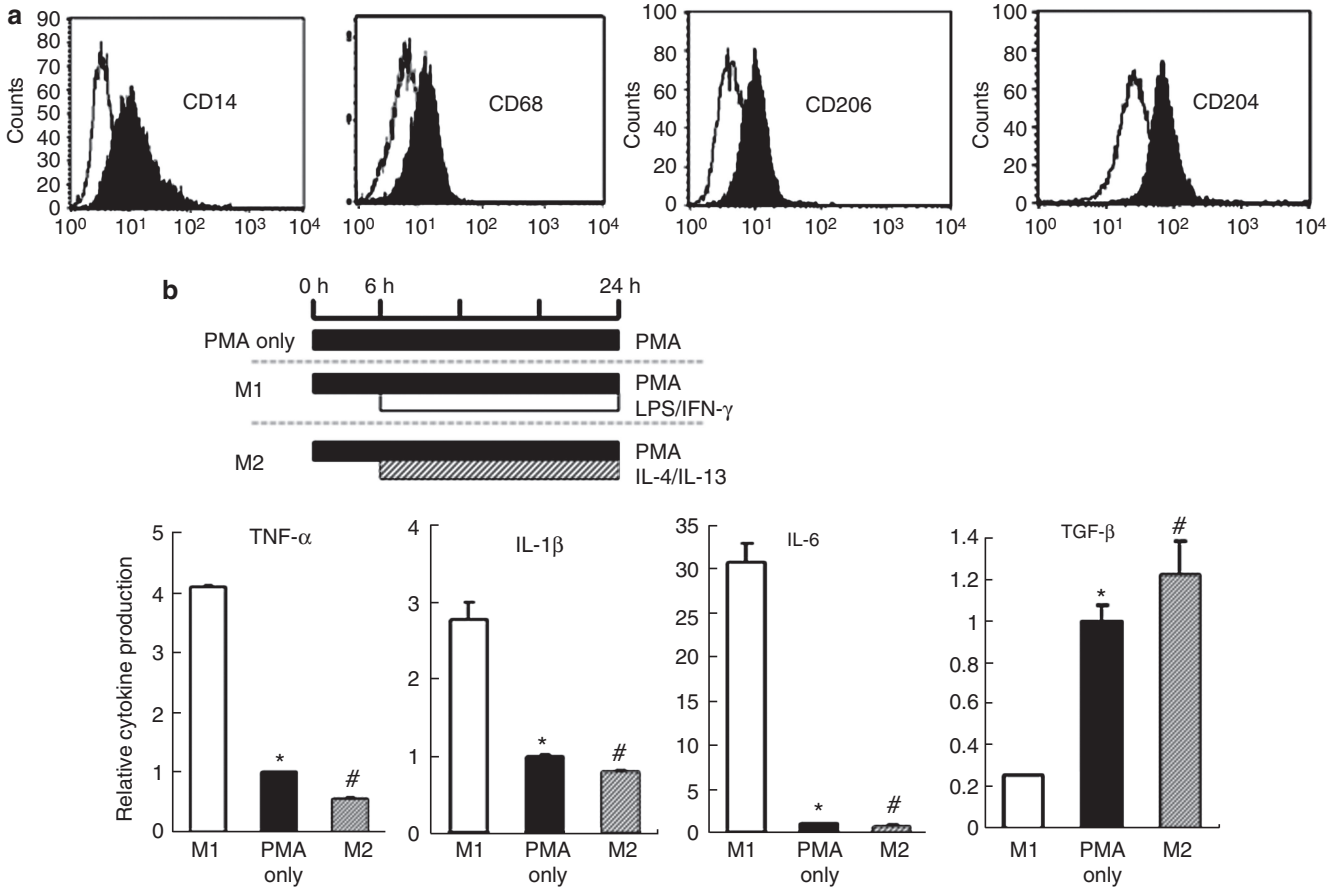

Figure 2. M2-polarized THP-1 macrophages induced COX-2-dependent invasion and angiogenesis of human BCC. (**a**) THP-1 cells treated with 320 nM PMA for 24 hours showed significant induction for CD14 (marker for monocyte differentiation), CD68 (marker for macrophages differentiation), CD206, and CD204 (both markers for M2 macrophages). (**b**) After treatment with 320 nM PMA for 24 hours, THP-1 cells differentiated to the ''PMA-treated THP-1 macrophages'' (labeled ''PMA only''). For the ''M1-polarized THP-1 macrophages'' (M1), THP-1 cells were treated with PMA for 6 hours and then cultured with PMA plus 100 ng ml^{-1} LPS and 20 ng ml^{-1} IFN-γ for the following 18 hours. For the ''M2-polarized THP-1 macrophages'' (M2), THP-1 cells were treated with PMA for 6 hours, then cultured with PMA plus 20 ng ml^{-1} IL-4 and 20 ng ml^{-1} IL-13 for another 18 hours. PMA-treated THP-1 macrophages (PMA only, black bars) and M2-polarized THP-1 macrophages (M2, hatched bars) both had significantly lower levels of TNF-α, IL-1β, and IL-6, and a higher level of TGF-β compared with that of M1-polarized THP-1 macrophages (M1, white bars; all $P<0.05$; *, PMA only vs M1; $^{\#}$ M2 vs M1). (**c**) For coculture experiments, 1×10^6 THP-1 cells were seeded into upper inserts (in a six-well plate) and treated with 320 nM PMA for 24 hours; these cells became ''PMA-treated THP-1 macrophages''. For ''M2-polarized THP-1 macrophages'', 1×10^6 THP-1 cells were seeded into upper inserts, treated with PMA for 6 hours, and then cultured with PMA plus 20 ng ml^{-1} IL-4/IL-13 for 18 hours. Both PMA-treated and M2-polarized THP-1 macrophages were washed thoroughly to remove all PMA and then cocultured with BCC cells (2×10^5 cells per well) in six-well plates for 6 hours. (**d**, **e**) BCC cells that had been cocultured with either PMA-treated THP-1 macrophages (black bars) or M2-polarized THP-1 macrophages (hatched bars) showed a significant increase in the number of invaded cells and tube-like structures (both $P<0.05$; *, PMA-treated THP-1 macrophages vs no coculture; $^{\#}$, M2-polarized THP-1 macrophages vs no coculture). The increments were abrogated by transient transfection of COX-2 siRNA or 30 minutes celecoxib (10 μM) treatment to BCC cells before they cocultured with both PMA-treated or M2-polarized THP-1 macrophages (all $P<0.05$; Δ, COX-2 siRNA vs coculture only; $^{\Theta}$, celecoxib 10 μM vs coculture only).

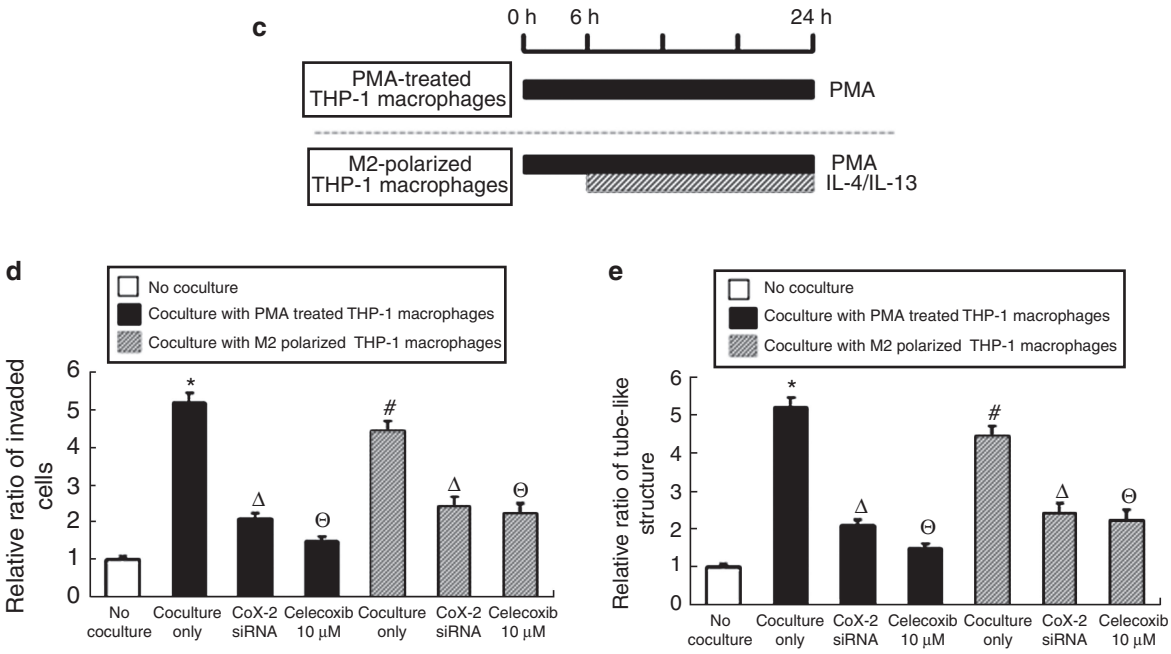

Figure 2. Continued.

evaluate the transcriptional regulation of COX-2, we transfected COX-2 promoter construct (−327/+59) or consensus sequence-mutated constructs into BCC cells and then cocultured BCC cells with THP-1 macrophages. We found that BCC cells transfected with nuclear factor-κB (NF-κB) consensus sequence mutated constructs, but not with nuclear factor NF-IL6- or cAMP-responsive element-mutated constructs, blocked luciferase activity induced by coculture (Figures 3b and Figure S2a). To determine which signaling pathway regulates NF-κB activity, BCC cells were transfected with NF-κB reporter plasmids and pretreated with specific inhibitors, including SB203580 (p38 MAPK inhibitor, 10 μM), PD98059 (ERK inhibitor, 25 μM), LY294002 (PI3K inhibitor, 25 μM), and SP600125 (JNK inhibitor, 10 μM) for 30 minutes before coculture with the PMA-treated THP-1 macrophages. Only pretreatment with SB203580 (10 μM) blocked NF-κB luciferase activity induced by coculture ($P<0.05$, Figures 3c and S2b). COX-2 mRNA induction was also consistently blocked by pretreatment of SB203580 (10 μM) and Bay 11-7082 (NF-κB inhibitor, 5 μM) (both $P<0.05$, Figure 3d). Taken together, these results suggest that macrophages induce COX-2 expression in BCC cells via the p38 MAPK/NF-κB cascade.

Degradation of the extracellular matrix by matrix metalloproteinases (MMPs) is important in BCC invasion (Kerkelä and Saarialho-Kere, 2003). We found that after coculture with the PMA-treated THP-1 macrophages, the number of invaded cells increased significantly in chambers coated with gelatin, but not fibronectin or collagen I (Figure 3e). This implies that macrophages induced gelatinase activity in BCC cells. We then found that macrophages induced significant secretion of MMP-9 (gelatinase-B), but not MMP-2 (gelatinase-A), from BCC cells (Figure 3f). When MMP-9 activity was blocked by a specific inhibitor before coculture, the number of invaded cells was significantly abrogated (Figure 3g). Taken together, macrophages might induce MMP-9 in BCC cells and subsequently increase the invasiveness of BCC cells.

Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor-A (VEGF-A) were previously identified as downstream effectors in COX-2-overexpressing BCC cells (Tjiu *et al.*, 2006). We found synergistic abrogation of *in vitro* angiogenesis by addition of bFGF and VEGF-A neutralizing antibodies into conditioned media (all $P<0.05$, Figure 3h). These results suggest that macrophages induce angiogenesis of BCC cells by VEGF-A and bFGF induction.

Monocyte-derived M2 macrophages also induced COX-2-dependent invasion and angiogenesis of BCC cells

To generalize our findings, we isolated monocytes from human peripheral blood mononuclear cells by magnetic cell sorting using CD14 microbeads. Macrophages were obtained by culturing monocytes (98% $CD14^+$) for 6 days in RPMI 1640 supplemented with 20% fetal calf serum (FCS) and 100 ng ml^{-1} macrophage colony-stimulating factor at a density of 1.5×10^5 cells per cm^2 (Martinez *et al.*, 2006). For M2 polarization, cells were then treated with 20 ng ml^{-1} IL-4 and 20 ng ml^{-1} IL-13 for 3 days (Martinez *et al.*, 2006); whereas for M1 polarization, macrophages were treated with 100 ng ml^{-1} LPS and 20 ng ml^{-1} IFN-γ for 3 days. Monocyte-derived M2 macrophages secreted low levels of TNF-α, IL-1β, and IL-6 and high level of TGF-β compared with their M1 counterparts (Figure S3). CD206 and CD204 were highly expressed on the cell surfaces of the ''monocyte-derived M2 macrophages'' (data not shown).

Two (2) $\times10^5$ monocyte-derived M2 macrophages were then seeded into upper inserts and cocultured with 2×10^5 BCC cells (in six-well plates) in serum-free RPMI 1640 for 24 hours. After coculture, macrophages (in upper inserts)

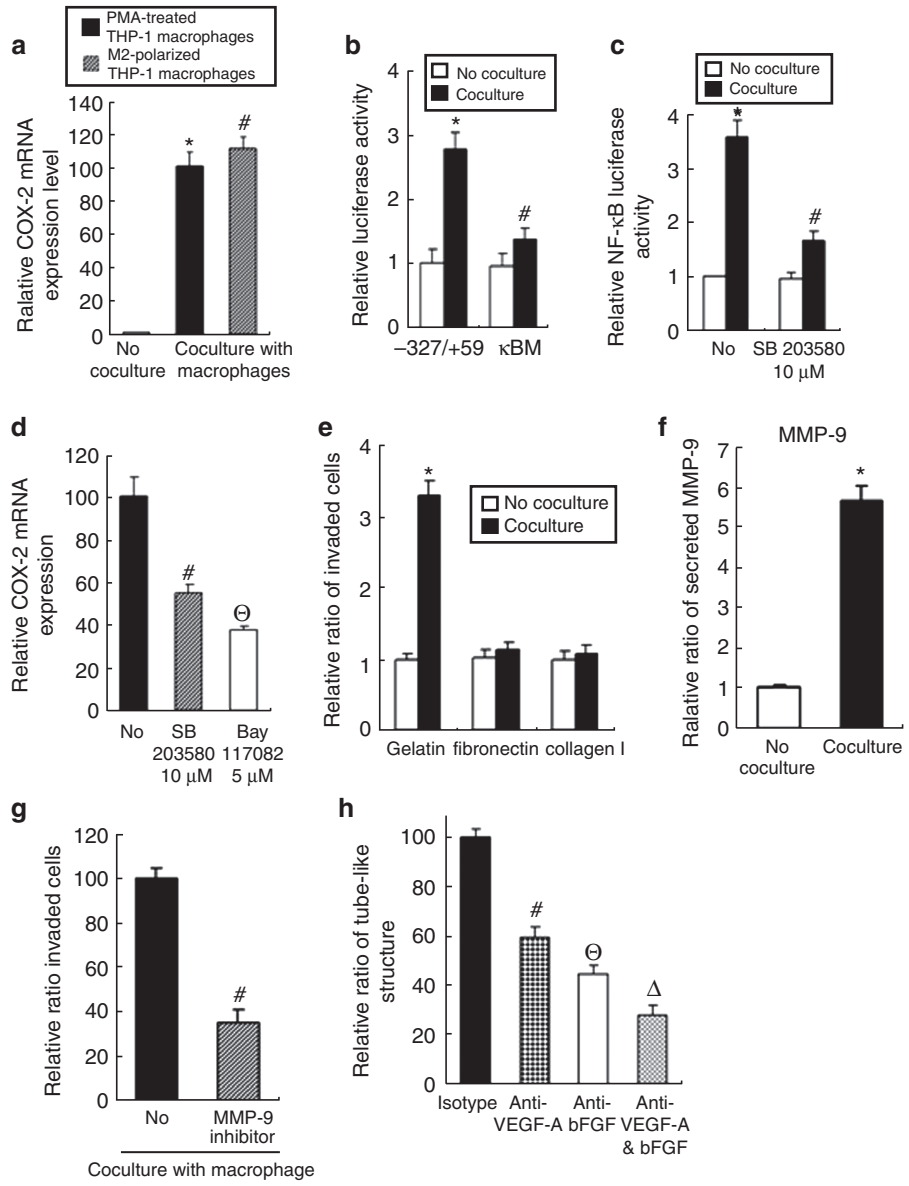

Figure 3. Macrophages activated a p38 MAPK/NF-κB/COX-2 cascade and induced MMP-9-dependent invasion and VEGF-A/bFGF-dependent angiogenesis in BCC cells. (**a**) After BCC cells were cocultured with PMA-treated THP-1 macrophages (black bar) or M2-polarized THP-1 macrophages (hatched bar) for 6 hours, real-time quantitative PCR revealed that COX-2 mRNA was significantly induced in BCC cells (both $P<0.05$; *, coculture with PMA-treated THP-1 macrophages vs no coculture; #, coculture with M2-polarized macrophages vs no coculture). (**b**) BCC cells were transiently transfected with COX-2 promoter luciferase constructs and then cocultured with PMA-treated THP-1 macrophages (black bars) for 6 hours. COX-2 promoter luciferase activity (−327/+59) was significantly induced by coculture ($P<0.05$; *, coculture vs no coculture). Transient transfection of BCC cells with NF-κB-binding site mutated constructs (κBM) abrogated coculture-induced COX-2 promoter activity ($P<0.05$; #, κBM vs −327/+59). (**c**) BCC cells were transiently transfected with NF-κB luciferase plasmids. Coculture with PMA-treated THP-1 macrophages (black bar) significantly induced NF-κB activity ($P<0.05$; *, coculture vs no coculture). Pretreating BCC cells with the p38 MAPK-specific inhibitor, SB203580 (10 μM), 30 minutes before coculture significantly abrogated NF-κB activity induced by coculture ($P<0.05$; #, SB203580 pretreatment vs no pretreatment). (**d**) BCC cells treated with p38 MAPK inhibitor (SB203580, 10 μM, hatched bar) or NF-κB inhibitor (Bay 11-7082, 5 μM, white bar) 30 minutes before coculture significantly abrogated COX-2 mRNA expression induced by PMA-treated THP-1 macrophages ($P<0.05$; #, SB203580 vs no inhibitor; Θ, Bay 117082 vs no inhibitor). (**e**) After coculture with PMA-treated THP-1 macrophages, BCC cells had increased numbers of invaded cells in chambers coated with gelatin ($P<0.05$; *, coculture vs no coculture), but not fibronection or collagen I. (**f**) Coculture with PMA-treated THP-1 macrophages also induced significant secretion of MMP-9 from BCC cells ($P<0.05$; * coculture vs no coculture). (**g**) MMP-9 specific inhibitor significantly abrogated macrophage-induced invasiveness of BCC cells ($P<0.05$; #, MMP-9 inhibitor vs no coculture). (**h**) Adding neutralizing antibodies to VEGF-A and bFGF synergistically abrogated *in vitro* angiogenesis (tube-like structures formation of HUVECs; all $P<0.05$; #, anti-VEGF-A vs no; Θ, anti-bFGF vs no; Δ, anti-VEGF-A/bFGF vs no).

were discarded. BCC cells were assayed for COX-2 mRNA, invasion and *in vitro* angiogenesis, as described previously. COX-2 mRNA was significantly induced at 24 hours after coculture with monocyte-derived M2 macrophages ($P<0.05$; Figure 4a). An increased number of invaded cells were found in BCC cells that had been cocultured with the monocyte-

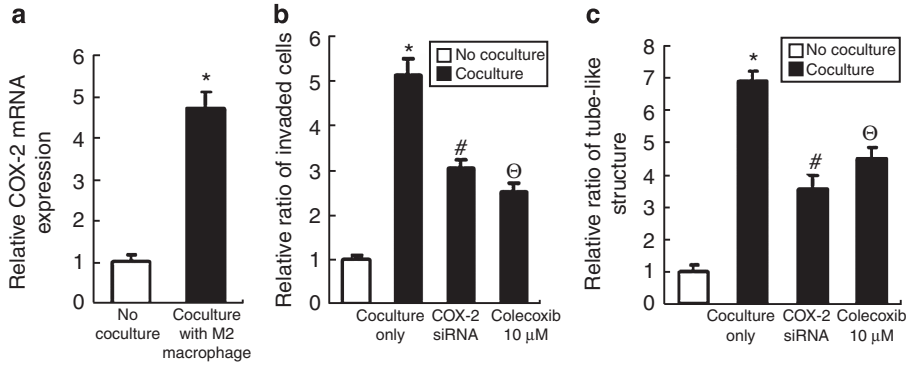

Figure 4. Monocyte-derived M2 macrophages also induced COX-2-dependent invasion and angiogenesis of human BCC cells. Monocyte-derived M2 macrophages were obtained from peripheral blood monocytes treated with 100 ng ml^{-1} M-CSF for 7 days, followed by 20 ng ml^{-1} IL-4 and 20 ng ml^{-1} IL-13 for 3 days. Monocyte-derived M2 macrophages (2×10^5) were seeded into upper inserts and cocultured with BCC cells (2×10^5 cells per well) in six-well plate for 24 hours. After coculture, macrophages were discarded. BCC cells were washed and assayed for COX-2 mRNA expression, invasion, and *in vitro* angiogenesis (HUVEC tube formation assay). Coculture induced significant COX-2 mRNA expression (**a**), invasion (**b**), and *in vitro* angiogenesis (**c**) ($P<0.05$; *, coculture vs no coculture). Pretreating BCC cells with COX-2 siRNA or celecoxib abrogated M2 macrophage-induced invasion and angiogenesis (all $P<0.05$; $^{\#}$, COX-2 siRNA vs coculture only; $^{\Theta}$, celecoxib vs coculture only).

derived M2 macrophages ($P<0.05$; Figure 4b). The increments were abrogated by COX-2 siRNA and celecoxib (10 μM) pretreatments (both $P<0.05$; Figure 4b). Conditioned media from BCC cells that had been cocultured with the monocyte-derived M2 macrophages resulted in increased number of tube-like structures of HUVECs ($P<0.05$; Figure 4c). The increase was abrogated by COX-2 siRNA and celecoxib (10 μM) pretreatments (both $P<0.05$; Figure 4c). To determine whether MMP-9, VEGF-A, and bFGF secretion from BCC cells were activated by coculture with monocyte-derived M2 macrophages. BCC cells that had been cocultured with monocyte-derived M2 macrophages were washed and serum-free media were added. Serum-free culture supernatants were collected at 24 hours and assayed by ELISA. Coculture induced increased release of MMP-9, VEGF-A, and bFGF from BCC cells, which could be abrogated by pretreating BCC cells with COX-2 siRNA or celecoxib (10 μM; Figure S4). Taken together, coculture with monocyte-derived M2 macrophages also induced COX-2-dependent invasion and angiogenesis of BCC cells.

DISCUSSION

We found that TAMs can induce invasion and angiogenesis of human BCC. TAMs were derived from circulating monocytes that are recruited at the tumor site by chemotactic factors (Balkwill *et al.*, 2005). A tumor microenvironment abundant in Th2 cytokines facilitated BCC progression (Kaporis *et al.*, 2007). However, the Th1 immune response induced by a topical immune modifier (imiquimod) caused BCC regression (Wenzel *et al.*, 2005). We focused on the effect of TAMs on cancer cells, and limited this study to effects mediated by paracrine regulation. We used a noncontact system described previously for coculture macrophages and BCC cells (Chen *et al.*, 2003, 2005). THP-1 macrophages were used as a macrophage model because PMA-treated THP-1 macrophages had an M2 functional profile (Chen *et al.*, 2003, 2005).

No PMA-containing macrophages or media were added to the coculture. PMA used to differentiate THP-1 cells was discarded and the PMA-treated cells were thoroughly washed (three times in PBS) to remove all PMA before they cocultured with BCC cells. To rule out the possibility that BCC cells might have been activated by trace PMA contamination, we fixed the PMA-treated THP-1 macrophages with 4% paraformaldehyde for 30 minutes at 4 °C. The paraformaldehyde-fixed (killed) macrophages washed with the same protocol, and then cocultured with BCC cells. We found that once the macrophages were killed, they could no longer induce invasion, *in vitro* angiogenesis, or COX-2 expression in BCC cells (Figure S5). If BCC cells had been activated by trace contamination of PMA directly, we would have seen significant induction of invasion, angiogenesis, and COX-2 expression, even after macrophage activities were ablated. This suggests that BCC cells were activated by THP-1 macrophage activity instead of contamination by PMA.

To further confirm that BCC cells were activated by THP-1 macrophage activity, we replaced THP-1 cells with primary human skin fibroblasts. All the experimental protocols were the same, except that THP-1 cells were replaced by fibroblasts. BCC cells cocultured with PMA-treated fibroblasts did not show significant induction of invasion, *in vitro* angiogenesis, or COX-2 expression comparing with BCC cells cocultured with fibroblasts without PMA treatment (Figure S5). Had the BCC cells been activated by PMA contamination, coculture with PMA-treated fibroblasts should have induced similar responses in invasion, angiogenesis, and COX-2 expression even without macrophages. However, our results suggest that the presence of THP-1 macrophages was essential for BCC cell activation. This argued against the conjecture that BCC cells were activated directly by PMA.

The truly PMA-free ''monocyte-derived M2 macrophages'' induced the same responses in BCC cells as the ''PMA-treated THP-1 macrophages'' did. These findings ruled out the possibility that BCC cells were activated directly by trace PMA contamination.

TAMs can directly activate tumor-promoting genes in cancer cells (Chen *et al.*, 2003, 2005). Consistent with previous

reports, we found that COX-2 mRNA induction by macrophages was highly regulated by the binding of NF-κB to its consensus sequences in the promoter region (Chang *et al.*, 2004). Macrophages also induce invasiveness via NF-κB in breast and ovarian cancers (Hagemann *et al.*, 2005). Macrophages activate p38 MAPK/NF-κB/COX-2 cascades in BCC cells, as corroborated by a previous report (Hung *et al.*, 2004).

The expression of MMP-1, −2, −3, −7, −9, −10, and 13 was previously reported in human BCC (Kerkelä and Saarialho-Kere, 2003). The reverse transcription–PCR results showed that only MMP-9 (and no other MMPs) was induced in BCC cells after coculture with macrophages (data not shown). Macrophages also activate MMP-9 activity in lung and breast cancer cells (Hagemann *et al.*, 2004; Chen *et al.*, 2005). In this report, we found macrophage-induced secretion of MMP-9 and MMP-9 enzymatic activity (detected by zymography) from BCC cells, which were blocked by p38 MAPK/NF-κB/COX-2 inhibition (Figure S6). Clinically, we detected MMP-9 expression in BCC cancer cells by immunohistochemistries (Figure S7). We found higher grade MMP-9 expression in BCC cancer cells, which correlated with increased depth of invasion, the number of TAMs, and epithelial COX-2 expression in BCC (all $P<0.05$, Table S1). Taken together, it appears that MMP-9 is responsible for increased invasion induced by macrophages.

Angiogenesis is essential to invasion and metastasis of many human cancers. Although BCC is a nonmetastatic tumor, formation of new vessels is also essential for the invasion of human BCC. We found that higher MVD correlated with deeper tumor invasion in human BCC cells ($\rho=0.7144$, $P<0.0001$, Spearman's rank correlations test). We previously reported that COX-2 overexpression induced bFGF and VEGF-A expression (Tjiu *et al.*, 2006). Here, we also demonstrated that these two angiogenic factors are important in TAM-induced angiogenesis of human BCC cells. In addition, *in vitro* angiogenesis, as well as VEGF-A and bFGF release from BCC cells after activation by macrophage coculture, was regulated by p38 MAPK/NF-κB/COX-2 cascades (Figure S8). bFGF and VEGF-A were also detected in human BCC cancer cells, further highlighting their clinical significance (Oh *et al.*, 2003).

In summary, we found that TAMs increased the invasion and angiogenesis of human BCC. Macrophages could induce BCC cells to release MMP-9, VEGF-A, and bFGF to support tumor cell invasion and angiogenesis in a COX-2-dependent manner. The targeting of TAMs as a therapeutic approach has been investigated in breast cancer and melanoma (Luo *et al.*, 2006; Gazzaniga *et al.*, 2007). Targeting TAMs or polarizing TAMs toward tumor-killing phenotypes by immune modulation may be a nonsurgical method to treat human BCC in the near future.

MATERIALS AND METHODS

Patients and specimens

The medical ethical committee of the National Taiwan University Hospital approved all described studies. The study was conducted according to Declaration of Helsinki Principles. Participants gave their written informed consent. A total of ninety-two patients (92 specimens), who received surgical excision of a BCC in the Department of Dermatology, National Taiwan University Hospital from 1999 to 2004, were enrolled in this study. Archived paraffin-embedded sections were collected from the Department of Pathology, National Taiwan University Hospital. Clinical characteristics were collected retrospectively by reviewing the patients' medical records.

Immunohistochemistry and evaluation of COX-2 expression, microvessel density, macrophage density, and depth of invasion

Using a standard ABC technique, antihuman COX-2 antibody (1:40 dilution; Cayman Chemical, Ann Arbor, MI), CD31 antibody (1:100 dilution; Dako, Carpinteria, CA), CD68 antibody (1:100 dilution; Dako), and MMP-9 (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) were applied on 92 BCC paraffin sections and localized with 3,3-diaminbenzidine tetrahydrochloride, followed by counterstaining with hematoxyline. COX-2 and MMP-9 were scored by a semiquantitative system, that is grade 0, <10%; grade 1, 10–50%; grade 2 >50% positive cells. MVD were evaluated by averaging the number of microvessels found in 10 selected high-power fields ($\times 200$ magnification) where the most CD31 staining was seen. The number of TAMs was determined by averaging the number of TAMs in 10 high-power fields where the most CD68 staining was observed. The depth of invasion (mm) was determined as the vertical distance from the deepest lower margin to the epidermis of the BCC.

Cell preparations

The human BCC cell line was tested free of mycoplasma and other trivial contaminants. THP-1 cells were obtained from ATCC (Manassas, VA). Both BCC and THP-1 cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FCS, 100 mg ml^{-1} penicillin, and 100 mg ml^{-1} streptomycin.

To generate PMA-treated THP-1 macrophages, 1×10^6 THP-1 cells were seeded into the upper insert of a six-well Transwell apparatus (0.4 μm pore size, Corning, Lowell, MA) and treated with PMA (320 nM) for 24 hours (Figure S1). To generate M2-polarized THP-1 macrophages, THP-1 cells were treated with 320 nM PMA for 6 hours, and then cultured with PMA plus 20 ng ml^{-1} IL-4 and 20 ng ml^{-1} IL-13 for another 18 hours (Figure S1). To generate M1-polarized THP-1 macrophages, THP-1 cells were treated with 320 nM PMA for 6 hours and then cultured with PMA plus 100 ng ml^{-1} LPS and 20 ng ml^{-1} IFN-γ for 18 hours.

Monocyte-derived M2 macrophages were generated as described previously (Martinez *et al.*, 2006). Briefly, human peripheral blood mononuclear cells were isolated using density gradient centrifugation. Monocytes (>98% purity) were isolated using anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Macrophages were obtained by culturing monocytes for 6 days in RPMI 1640 supplemented with 20% FCS and 100 ng ml^{-1} macrophage colony-stimulating factor at a density of 1.5×10^5 per cm^2. Macrophage polarization was obtained by removing the culture media and culturing cells for an additional 72 hours in RPMI 1640 supplemented with 5% FCS and 100 ng ml^{-1} LPS plus 20 ng ml^{-1} IFN-γ (for M1 polarization) or 20 ng ml^{-1} IL4 plus 20 ng ml^{-1} IL-13 (for M2 polarization).

Human skin fibroblasts were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 25 mM Hepes, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 10% FCS at 37 °C in a 5% CO_2 humidified incubator.

Macrophage and BCC cell coculture

After a thorough wash to remove all PMA, PMA-treated THP-1 macrophages or M2-polarized THP-1 macrophages (upper inserts) were cocultured with BCC cells (in a six-well plate, 2×10^5 cells per well) without direct contact (Figure S1). After 6 hours of coculture, upper inserts containing macrophages were discarded, and BCC cells were washed and then used for subsequent experiments.

Monocyte-derived M2 macrophages (2×10^5) were seeded into upper inserts and cocultured with BCC cells (2×10^5 cells per well) in a six-well plate. After 24 hours of coculture, the upper inserts were discarded, and BCC cells washed and used for subsequent experiments.

Flow cytometry

After blocking human FcRs, cells were washed and resuspended in PBS supplemented with 1% heat-inactivated FBS and 0.01% NaN_3. For CD68 staining, cells were fixed and permeabilized with a BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD Biosciences, Franklin Lakes, NJ). Cells were then incubated with the FITC-CD68 mAb (Dako). For surface markers, cells were incubated with FITC-CD14 mAb (BD Biosciences), or PE-CD206 mAb (BD Biosciences). For detection of CD204, cells were incubated with antihuman CD204 mAb (R&D systems, Minneapolis, MN), washed, and labeled with PE-conjugated goat anti-mouse secondary antibody. Following the final washing step, labeled cells were analyzed by flow cytometry on a FACScan flow cytometer using CellQuest software (BD Biosciences).

Plasmid transient transfection and luciferase activity assay

The COX-2 promoter (−327/+59), NF-κB (−223/−214)-binding site mutated, NF-IL6-binding site (−132/−124) mutated, or cAMP-responsive element-binding site (−59/−53) mutated constructs were obtained from Dr Inoue (Nara University, Nara, Japan). The NF-κB luciferase reporter was obtained from Clontech (Mountain View, CA). BCC cells were transfected with COX-2 promoter constructs (1 μg) or NF-κB luciferase reporter (1 μg) along with β-galactosidase DNA (1 μg, Clontech) using Lipofectamine (Invitrogen). After transient transfection, BCC cells were serum-deprived for 24 hours and cocultured with THP-1 macrophages for 6 hours. Cell extracts were then prepared and the luciferase and β-galactosidase activities were measured. The luciferase activity was normalized to the β-galactosidase activity.

COX-2-specific RNA interference

The target sequence for the *COX-2* siRNA was bases 291–313 of NM000963.1. (5′-aactgctcaacaccggaattttt-3′; Denkert *et al.*, 2003). The *COX-2* siRNA sequence was cloned into the pRNA-U6/neo constitutively expressing vector (GenScript, Piscataway, NJ). The COX-2 siRNA and control vectors were transiently transfected into BCC cells before they were cocultured with macrophages.

Detection of the cytokine profile of macrophages

Culture supernatants were collected from PMA-treated THP-1 macrophages or M1/M2-polarized THP-1 macrophages at 24 hours. Culture supernatants were collected from monocyte-derived M1/M2 macrophages 72 hours after either LPS/IFN-γ (M1) or IL-4/IL-13 (M2) were added to the macrophages. The cytokine levels were measured with TNF-α, IL-1β, IL-6, and TGF-β EIA kits (all from R&D Systems).

Detection of VEGF-A, bFGF, and MMP-9 released from BCC cells

BCC cells were cocultured with either PMA-treated THP-1 macrophages for 6 hours or monocyte-derived M2 macrophages for 24 hours. After coculture, macrophages were discarded. BCC cells were washed and serum-free RPMI 1640 were added to BCC cells. The serum-free supernatants were collected at 24 hours and subjected to ELISA assays for VEGF-A, bFGF, and MMP-9.

In vitro angiogenesis assay

After BCC cells were cocultured with THP-1 macrophages for 6 hours or monocyte-derived M2 macrophages for 24 hours, macrophages were removed. BCC cells were washed and fresh media added. After 24 hours, collected culture supernatants were designated as conditioned media and concentrated 10-fold. HUVECs were isolated and cultured as previously described (Jee *et al.*, 2004). HUVECs (1×10^4 cells in 50 μl per well) were seeded into a Matrigel-coated (50 μl per well) 96-well plate. Then, 50 μl of 10-fold concentrated conditioned media were added to the HUVECs and incubated for 6 hours at 37 °C. Tube-like structures of HUVECs formed at 6 hours were counted under a microscope.

Invasion assay

The invasion assay was conducted using Transwell cell culture chambers (24 wells, 8 μm pore size; Corning). Briefly, upper inserts were coated with 50 μl of different extracellular matrix components, including 10 mg ml^{-1} Matrigel (BD Biosciences), 0.2 mg ml^{-1} gelatin (Sigma; St Louis, MO, USA), 0.1 mg ml^{-1} fibronectin (Sigma), or 0.2 mg ml^{-1} collagen I (Sigma), and allowed to set for 1 hour at 37 °C. BCC cells were harvested after 6 hours of coculture with PMA-treated THP-1 macrophages or 24 hours of coculture with monocyte-derived M2 macrophages. Then, 1×10^5 BCC cells were resuspended in 200 μl of RPMI 1640 supplemented with 10% FCS, and added to the upper inserts. RPMI 1640 (500 μl) with 10% FCS was added to the lower chamber. After BCC cells attached, the media (upper and lower chambers) were changed to serum-free RPMI 1640. At 24 hours later, invaded cells were fixed and stained with 0.05% crystal violet. Cells on the upper surface of the insert membrane were removed with cotton rods. The invaded cells were counted at ×200 magnification in 10 different fields for each insert. The experiments were repeated three times.

Antibodies and reagents

See Supplementary materials and methods.

Real-time quantitative PCR

See Supplementary materials and methods.

Zymography

See Supplementary materials and methods.

Statistical methods

Tests for trends were used to examine trends among the ordered groups. The Wilcoxon rank-sum test was used to compare nonparametric continuous variables of different groups. The Kruskal–Wallis test was used to determine the association between categorical variables. Multivariate linear regression was applied to test independent influencing factors. All tests were two-sided, and a

P-value of <0.05 was considered statistically significant. All statistical tests were performed using STATA 8.0 software (StataCorp, College Station, TX).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by grants from the National Science Council, Executive Yuan, Taiwan (NSC 96-2314-B-002-171-MY3, NSC 95-2314-B-002-120-MY2, NSC 97-2314-B-002-122), and National Taiwan University Hospital (95-M12, 97-S839, 97-M958).

SUPPLEMENTARY MATERIAL

Supplementary Materials and Methods

Table S1. Increased epithelial MMP-9 expression was associated with increased depth of invasion, number of TAM, and epithelial COX-2 expression in BCC.

Figure S1. Experimental protocols for generating PMA-treated THP-1 macrophages, M2-polarized THP-1 macrophages and coculture experiments.

Figure S2. PMA-treated THP-1 macrophages induced COX-2 expression in BCC cells via p38 MAPK and NF-κB activation.

Figure S3. Monocyte-derived M2 macrophages secrete low TNF-α, IL-1β, and IL-6 and high TGF-β.

Figure S4. Coculture with monocyte-derived M2 macrophages induced COX-2-dependent MMP-9, VEGF-A, bFGF release from BCC cells.

Figure S5. BCC cells were activated by macrophage activity but not directly by PMA.

Figure S6. MMP-9 secretion and enzymatic activity induced by coculture with PMA-treated THP-1 macrophages was regulated by p38 MAPK/NF-κB/COX-2 signaling cascades.

Figure S7. MMP-9 was strongly expressed in epithelial cells of aggressive subtypes of human BCC.

Figure S8. *In vitro* angiogenesis, VEGF-A, and bFGF secretion induced by coculture with PMA-treated THP-1 macrophages were regulated by the p38 MAPK/ NF-κB/COX-2 pathway.

REFERENCES

Balkwill F, Carles KA, Mantovani A (2005) Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* 7:211–7

Cervello M, Foderàa D, Florena AM, Soresi M, Tripodo C, D'Alessandro N *et al.* (2005) Correlation between expression of cyclooxygenase-2 and the presence of inflammatory cells in human primary hepatocellular carcinoma: possible role in tumor promotion and angiogenesis. *World J Gastroenterol* 11:4638–43

Chang YJ, Wu MS, Lin JT, Sheu BS, Muta T, Inoue H *et al.* (2004) Induction of cyclooxygenase-2 overexpression in human gastric epithelial cells by *Helicobacter pylori* involves TLR2/TLR9 and c-Src-dependent nuclear factor-kappaB activation. *Mol Pharmacol* 66:1465–77

Chen JJ, Lin YC, Yao PL, Yuan A, Chen HY, Shun CT *et al.* (2005) Tumor-associated macrophages: the double-edged sword in cancer progression. *J Clin Oncol* 23:953–64

Chen JJ, Yao PL, Yuan A, Hong TM, Shun CT, Kuo ML *et al.* (2003) Up-regulation of tumor interleukin-8 expression by infiltrating macrophages: its correlation with tumor angiogenesis and patient survival in non-small cell lung cancer. *Clin Cancer Res* 9:729–37

Denkert C, Fürstenberg A, Daniel PT, Koch I, Köbel M, Weichert W *et al.* (2003) Induction of G0/G1 cell cycle arrest in ovarian carcinoma cells by the anti-inflammatory drug NS-398, but not by COX-2-specific RNA interference. *Oncogene* 22:8653–61

Gazzaniga S, Bravo AI, Guglielmotti A, van Rooijen N, Maschi F, Vecchi A *et al.* (2007) Targeting tumor-associated macrophages and inhibition of MCP-1 reduce angiogenesis and tumor growth in a human melanoma xenograft. *J Invest Dermatol* 127:2031–41

Hagemann T, Robinson SC, Schulz M, Trümper L, Balkwill FR, Binder C (2004) Enhanced invasiveness of breast cancer cell lines upon co-cultivation with macrophages is due to TNF-alpha dependent up-regulation of matrix metalloproteinases. *Carcinogenesis* 25:1543–1549

Hagemann T, Wilson J, Kulbe H, Li NF, Leinster DA, Charles K *et al.* (2005) Macrophages induce invasiveness of epithelial cancer cells via NF-kappa B and JNK. *J Immunol* 175:1197–205

Hung JH, Su IJ, Lei HY, Wang HC, Lin WC, Chang WT *et al.* (2004) Endoplasmic reticulum stress stimulates the expression of cyclooxygenase-2 through activation of NF-kappaB and pp38 mitogen-activated protein kinase. *J Biol Chem* 279:46384–92

Jee SH, Chu CY, Chiu HC, Huang YL, Tsai WL, Liao YH *et al.* (2004) Interleukin-6 induced basic fibroblast growth factor-dependent angiogenesis in basal cell carcinoma cell line via JAK/STAT3 and PI3-kinase/Akt pathways. *J Invest Dermatol* 123:1169–75

Kaporis HG, Guttman-Yassky E, Lowes MA, Haider AS, Fuentes-Duculan J, Darabi K *et al.* (2007) Human basal cell carcinoma is associated with Foxp3+ T cells in a Th2 dominant microenvironment. *J Invest Dermatol* 127:2391–8

Kaur P, Mulvaney M, Carlson JA (2006) Basal cell carcinoma progression correlates with host immune response and stromal alterations: a histologic analysis. *Am J Dermatopathol* 28:293–307

Kerkelä E, Saarialho-Kere U (2003) Matrix metalloproteinases in tumor progression: focus on basal and squamous cell skin cancer. *Exp Dermatol* 12:109–25

Lewis CE, Pollard JW (2006) Distinct role of macrophages in different tumor microenvironments. *Cancer Res* 66:605–12

Luo Y, Zhou H, Krueger J, Kaplan C, Lee SH, Dolman C *et al.* (2006) Targeting tumor-associated macrophages as a novel strategy against breast cancer. *J Clin Invest* 116:2132–41

Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M (2004) The chemokine systems in diverse forms of macrophage activation and polarization. *Trends Immunol* 25:677–86

Mantovani A, Sozzani S, Locati M, Allavena P, Sica A (2002) Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* 23:549–555

Martinez FO, Gordon S, Locati M, Manotvani A. (2006) Transcriptional profiling of the human monocyte-to macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol* 199:7303–11

Miller DL, Weinstock MA (1994) Nonmelanoma skin cancer in the United States: incidence. *J Am Acad Dermatol* 30:774–8

Oh CK, Kwon YW, Kim YS, Jang HS, Kwon KS (2003) Expression of basic fibroblast growth factor, vascular endothelial growth factor and thrombospondin-1 related to microvessel density in nonaggressive and aggressive basal cell carcinoma. *J Dermatol* 30:306–13

Sica A, Schioppa T, Mantovani A, Allavena P (2006) Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. *Eur J Cancer* 42:717–27

Tjiu JW, Liao YH, Lin SJ, Huang YL, Tsai WL, Chu CY *et al.* (2006) Cyclooxygenase-2 overexpression in human basal cell carcinoma cell line increases antiapoptosis, angiogenesis, and tumorigenesis. *J Invest Dermatol* 126:1143–51

Walling HW, Fosko SW, Geraminejad PA, Whitaker DC, Arpey CJ (2004) Aggressive basal cell carcinoma: presentation, pathogenesis, and management. *Cancer Metastasis Rev* 23:389–402

Wang W, Bergh A, Damber JE (2005) Cyclooxygenase-2 expression correlates with local chronic inflammation and tumor neovascularization in human prostate cancer. *Clin Cancer Res* 11:3250–6

Watkins SK, Egilmez NK, Suttles J, Stout RD (2007) IL-12 rapidly alter the functional profiles of tumor-associated and tumor-infiltrating macrophages *in vitro* and *in vivo*. *J Immunol* 178:1357–62

Wenzel J, Uerlich M, Haller O, Bieber T, Tueting T (2005) Enhanced type 1 interferon signaling and recruitment of chemokine receptor CXCR3-expressing lymphocytes into the skin following treatment with the TLR7-agonist imiquimod. *J Cutan Pathol* 32:257–262