RESEARCH ARTICLE

Macrophages Promote Coal Tar Pitch Extract-induced Tumorigenesis of BEAS-2B Cells and Tumor Metastasis in Nude Mice Mediated by AP-1

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Abstract

Background: We sought to evaluate the role of tumor associated macrophages (TAMs) on the promotion of coal tar pitch extract (CTPE)-induced tumorigenesis of human bronchial epithelial cells (BEAS-2B) and tumor metastasis in nude mice, and related mechanisms. Materials and Methods: BEAS-2B cells were first treated with 2.4 mg/mL CTPE for 72 hours. After removal of CTPE, the cells were continuously cultured and passaged using trypsin-EDTA. THP-1 cells were used as macrophage-like cells. BEAS-2B cells under different conditions (n=6/group) were injected into the back necks of nude mice, and alterations of tumor xenograft growth, indicative of tumorigenicity, and tumor metastasis were determined. Pathological changes (tumor nests and microvascular lesions) of HE-stained tumor tissues were also evaluated. The expression of AP-1(c-Jun) in xenografts and metastatic tumors was determined using immunohistochemistry. Results: Tumor size and weight in nude mice transplanted with the mixture of CTPE-induced passage 30 BEAS-2B and THP-1 cells (2:1) were increased compared to those from the CTPE-treated BEAS-2B cells at passage 30 alone at different observation time points. Tumor metastasis to lymph nodes and liver was only detected after transplantation of a mixture the two kinds of cells. The numbers of tumor nests and microvascular lesions, and the expression levels of AP-1 (c-Jun) in tumors from the mixture of two kinds of cells were increased apparently in contrast to those in tumor from the CTPE-treated BEAS-2B cells of passage 30 alone. In addition, there was positive correlation between AP-1 (c-Jun) expression level and the number of microvascular lesions, or between AP-1 (c-Jun) expression level and tumor metastasis in these two groups. Conclusions: TAMs not only facilitate tumorigenesis transformation of CTPE-induced BEAS-2B cells, but also promote tumor growth, angiogenesis and metastasis in nude mice *in vivo*, which may be mediated by AP-1.

Keywords: Tumor associated macrophages - tumorigenesis - metastasis - lung cancer - coal tar pitch - AP-1

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Introduction

It has been becoming increasingly clear that tumor inflammatory microenvironment is the underlying cause of many types of cancer (Mantovani et al., 2008; Freire et al., 2013; Schauer et al., 2013). Mantovani and coworkers showed that the "smoldering" cancer-related inflammation is the additional seventh hallmarks of cancer (Hanahan and Weinberg, 2011). In sites of chronic inflammation, these "tumor associated macrophages (TAMs)" have been considered very important. There is accumulating evidence suggesting TAMs have a wide range of pro-tumor functions which effect on nearly every stage of tumor occurrence and development, such as tumor growth, angiogenesis, immune suppression, and metastasis (Hao et al., 2012; Menen et al., 2012; Owen and Mohamadzadeh, 2013). Human gastric carcinoma TMK-1 cells with monocyte chemoattractant protein-1 gene transfection were transplanted into nude mice, angiogenesis and tumorigenesis of gastric carcinoma subsequently occurred in nude mice via macrophage recruitment (Kuroda et al., 2005). In a Balb/C mouse model of mammary carcinoma, bone marrow-derived, alternatively activated macrophages enhance mammary tumor growth and lung metastasis, which were mediated by cytokines produced by the crosstalk of mammary carcinoma and macrophages: granulocyte colony-stimulating factor, IL-1a, IL-2, IL-16, et al (Cho et al., 2012). Zhang and colleagues demonstrated that macrophages promoted the metastatic behavior of Lewis lung carcinoma cell in vitro, using

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the co-culture Transwell system (Zhang et al., 2012). However, there have been no studies to show the role of TAMs in initiation, progression and metastasis of lung cancer in vivo thus far.

Activator protein 1 (AP-1) transcription factor is composed of Jun (c-Jun, JunB and JunD) and Fos (c-Fos, FosB, Fra1 and Fra2) subfamilies. Jun proteins can both homo- and heterdimerize, whereas Fos can't homodimerize. AP-1 with Jun proteins can bind DNA, therefore, c-Jun is the representative of AP-1. At present, increasing evidence has suggested AP-1 plays a role in inflammation, cellular proliferation and transformation, apoptosis, cellular migration (Wagner and Eferl, 2005; Eychene et al., 2008; Shaulian, 2010). AP-1 has similar function to NF-κB on regulating expression of cytokines, such as TNF-α and interleukin (Chu, 2013), which contribute to tumorigenesis and tumor development. For example, c-Jun was required in the development of chemical-induced hepatocellular cacinoma (Eferl et al., 2003).

Coal tar pitch (CTP) is a soft to hard and brittle substance containing chiefly aromatic resinous compounds along with aromatic and other hydrocarbons and their derivatives; it is used mainly as road tar, in waterproofing roofs and other structures, and to make electrodes. CTP is a recognized carcinogen, which can induce lung cancer selectively (Koganti et al., 2000; Williams et al., 2013). In previous study, we demonstrated that TAMs promoted CTP extract (CTPE)-induced tumorigenecity in vitro using co-culture system of human bronchial epithelial cells (BEAS-2B) and macrophage-like THP-1 cells (Feng et al., 2012).

In this study, we evaluated the role of macrophages in the promotion of lung cancer progression and metastasis using a nude mice model that is extensively used as an in vivo tumor model, in which CTPE-induced transformed BEAS-2B cells were injected into nude mice in conjunction with macrophage-like THP-1 cells. Furthermore, we investigated the expression levels of c-Jun in xenograft tumor of nude mice, and analyzed the correlation between c-Jun protein expression levels and angiogenesis, metastasis to explore the implicated mechanism.

Materials and Methods

Preparation of coal tar pitch extract (CTPE)

CTPE was collected as described previously (Feng et al., 2012). Dimethyl sulfoxide (DMSO) (spectroscopically pure) was added to get the extract solution. The stock concentration of CTP extract was 2 mg/mL.

Cell lines and cell culture

Human bronchial epithelial cell line (BEAS-2B): The BEAS-2B cell line was derived by transforming human bronchial epithelial cells with an adenovirus 12-simian virus 40 construct. Human macrophage-like cell line (THP-1) was purchased from ATCC (Rockville, USA). THP1 cells display several characteristics of tumor associated macrophages (TAMs), such as defective activation of NF-κB, lack of nitric oxide production in response to LPS/IFNγ and high constitutive STAT1 signaling (Kaler et al., 2009). The two cell lines were cultured in RPMI 1640 medium with 10% (v:v) of fetal bovine serum (FBS), 100 IU/mL of penicillin and 100 mg/mL of streptomycin. All the cells were cultured at 37°C in a 5% CO_2 incubator.

CTPE treatment

BEAS-2B cells were treated with 2.4 μg/mL CTPE (30% of IC_{50}) for 72 h. Grown to 70%-80% confluence, the BEASS-2B cells were treated with CTPE solution for 24 h. After removal of CTPE, the cells were washed with cold PBS and passaged using trypsin-EDTA; this same process was repeated three times for a total of 72 h.

0.2% DMSO was used as vehicle control. For simplification, the passage of BEAS-2B cell was numbered as passage 0 following CTPE treatment.

Tumor formation in nude mice

SPF degree 3~4-week-old male BALB/C nude mice were bought from Slac Laboratory Animal Company, Hunan, China, and fed in the Medical Animal Center in Zhengzhou University. Nude mice were grouped randomly, and BEAS-2B cells under different conditions (blank control, DMSO treatment, passage 20 and passage 30 of BEAS-2B cells after CTPE treatment, and the mixture of the CTPE-induced BEAS-2B cells at passage 30 and THP-1 cells (2:1)) in 200 μL PBS were injected into the back neck of isoflurane-anesthetized nude mice (n=6 for each group). The total cell number transplanted into each mouse was 2×10^7. The condition of the mice was monitored every other day after transplantation, and the size of the tumor was estimated according to the formula $V= 1/2\times L\times W^2$ (L represented tumor length, and W tumor width) every 5 days after inoculation. When the animals were finished the observation of 30 days, the mice were humanely euthanized with CO_2, and the tumors were removed and weighted on the 30th day. All processes were performed with nude mice in accordance with the guidelines of Zhengzhou University for animal experiments. And the Life Sciences Institutional Review Board of Zhengzhou University approved this study.

Counts of tumor nests and microvasculars in tumor slides

Pathological changes of HE-stained tumor slides were observed, including atypical cells with dark-stained nuclear, tumor nest and microvascular. Tumor nest, which is an isolated collection or clump of tumor cells in tissue of a different structure with clear boundary, and microvascular which cross-section contains more than 10 red blood cells were observed using microscope. The numbers of tumor nests and microvasculars were counted in 10 randomly-selected high power vision fields under microscope.

Immunohistochemistry (IHC)

Briefly, tumors on slides were blocked with goat serum for 30 min, and incubated with mouse anti-mouse c-Jun monoclonal antibody (Santa Cruz, 1:50 dilution) overnight at 4oC, then incubated with biotinylated rabbit anti-mouse immnunoglobulin at a concentration of 1:100 at 37°C

for 30 min. Positive IHC staining of c-Jun expression was reflected as the brown staining in the cytoplasm and estimated by mean density in 10 high power vision fields using Image-Pro Plus 6.0

Statistics

Data are expressed as mean ± SEM, SPSS12.0 (IBM, NC, USA) were used for statistical analysis. Significant difference between two groups with one variant was determined by Student t test. The correlation analysis was used for correlation between two variants. A two-tailed P value < 0.05 was considered statistically significant.

Results

Tumor xenograft occurrence and growth in nude mice

Tumor formation in nude mice resembles tumorigenicity in vivo. Two tumors were observed on the neck back of nude mice transplanted with the mixture of CTPE-treated BEAS-2B cells at passage 30 and THP-1 cells on the 10th day after transplantation, four tumors were observed on the 15th day, and all of six tumors were formed on the 20th day. After CTPE-treated BEAS-2B cells of passage 30 were transplanted in the neck back of nude mice, the first two tumors were observed on the 15th day and three tumors on 20th day. Total six tumors were observed by the 30th day (Figure 1A). BEAS-2B cells without treatment were used as negative control (blank); BEAS-2B cells treated by DMSO were used as vehicle control. 30 days following cell transplantation, there was no tumor formation in blank, DMSO, or CTPE group at passage 20.

Status of nude mice after transplantation

There was no tumor formation in nude mice in blank, DMSO, and CTPE group at passage 20. However, tumor occurred in all six nude mice transplanted with CTPE-induced BEAS-2B cells of 30 passage and with the mixture of CTPE-treated BEAS-2B cells at passage 30 and THP-1 cells. The mice didn't die during the experimental period. When the observation of 30 days was done, the mice were humanely euthanized with CO2. Tumors were peeled off and weighed on the 30th day after transplantation. The average weight of tumors derived from CTPE-induced passage 30 BEAS-2B/THP-1 cells was 1.942±0.628g, which was increased compared to the CTPE-treated BEAS-2B cells of passage 30 alone (0.532±0.441g) (P<0.05) (Figure 1B).

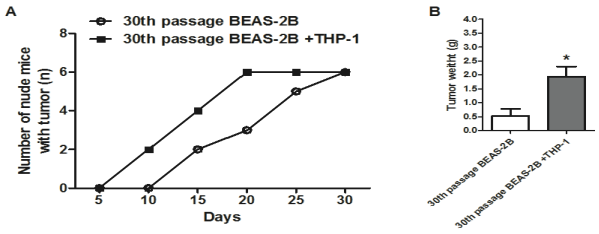

Figure 1. Tumor Xenograft Occurrence and Growth in Nude Mice. (A) Number of nude mice with tumor every five days after transplantation; (B) The average weights of tumors from CTPE-induced passage 30 BEAS-2B/THP-1 cells was increased compared to the CTPE-treated BEAS-2B cells of passage 30 alone (*vs CTPE-treated BEAS-2B cells of passage 30, p<0.05)

Pathological changes of xenografts

Pathological alterations of xenografts were observed using HE staining, most cells were stained with deep-stained nuclei and increased nuclear cytoplasmic ratio (Figure 2A-c, d: blue arrow), tumor nests (Figure 2A-a, b: red arrow) and microvasculars (Figure 2A-c, d: yellow arrow) were observed in tumors from CTPE-induced passage 30 BEAS-2B/THP-1 cells and the CTPE-treated BEAS-2B cells of passage 30 alone. However, the numbers of tumor nests and microvasculars in tumors from CTPE-induced passage 30 BEAS-2B/THP-1 cells were 7.00±0.97 and 20.17±2.64 respectively, which were increased apparently compared to those in tumor from the CTPE-treated BEAS-2B cells of passage 30 alone that were 3.50±0.56 and 7.00±1.03, the differences were statistically significant (P<0.05) (Figure 2B, C).

Tumor metastasis after transplantation of CTPE-induced passage 30 BEAS-2B/THP-1 cells

On the 30th day after cell transplantation, nude mice were sacrificed and organs observed, and we observed tumors metastasis to lymph nodes and liver after transplantation of CTPE-induced passage 30 BEAS-2B/THP-1 cells: cervical lymph nodes enlarged in three nude mice of total six nude mice (Figure 3A-a, b), and there were grain-like particles in livers in two mice of total six mice (Figure 3A-c, d).

Pathological alterations of metastasis tumors were observed using HE staining, and most cells were necrotic in enlarged lymph nodes (Figure 3B-a), a part of sinusoidal disappeared and lots of abnormal cells with deep-stained nuclei showed in metastasis livers (Figure 3B-b).

Expression of c-Jun in xenografts and metastasis tumors

c-Jun was detected in cytoplasm of tumor cells with brown staining (Figure 4A). The mean density of cells with positive c-Jun staining in xenograft tumors

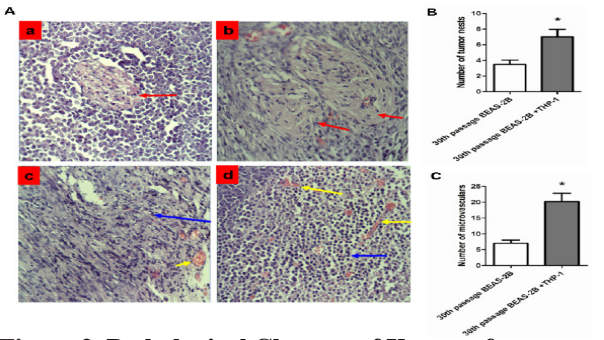

Figure 2. Pathological Changes of Xenografts. (A-a, c) Pathological changes of tumors transplanted with CTPE-treated BEAS-2B cells of passage 30 alone (400×); (A-b, d) Pathological changes of tumors from CTPE-induced passage 30 BEAS-2B/THP-1 cells. Red arrows: tumor nests; Blue arrows: abnormal cells with deep-stained nuclei and increased nuclear cytoplasmic ratio; Yellow arrows: microvasculars. B and C showed that the numbers of tumor nests and microvasculars in tumors from CTPE-induced passage 30 BEAS-2B/THP-1 cells were increased significantly compared to those in tumor from the CTPE-treated BEAS-2B cells of passage 30 alone, respectively. (*vs CTPE-treated BEAS-2B cells of passage 30, p<0.05)

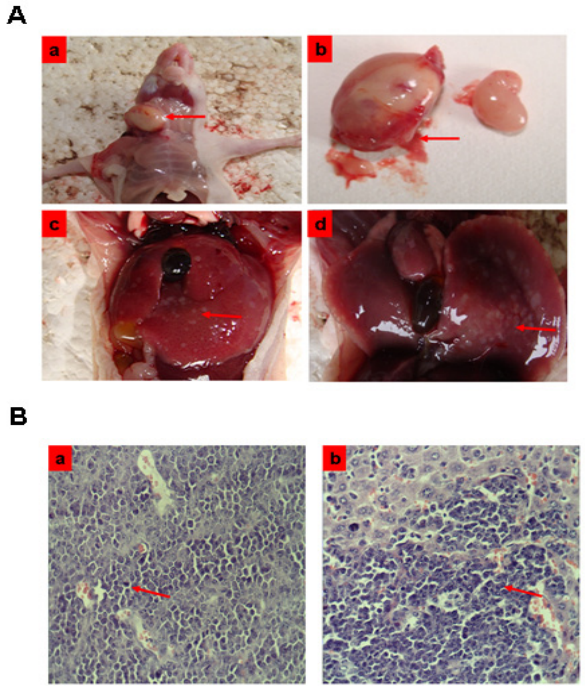

Figure 3. Tumor Metastasis after Transplantation of CTPE-induced Passage 30 BEAS-2B/THP-1 Cells. (A-a, b) The representatives of metastatic lymph node; (A-c,d) The representatives of metastatic liver; (B-a) Pathological changes of metastatic lymph node (400×);(B-d) Pathological changes of metastatic liver (400×)

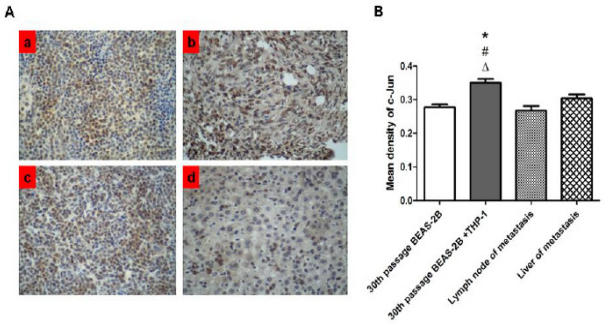

Figure 4. c-Jun Protein Expression Levels in Xenografts and Metastasis Tumors of Nude Mice. (A) c-Jun protein expression levels in xenografts and metastasis tumors of nude mice using IHC. (A-a) xenograft tumor from CTPE-treated BEAS-2B cells of passage 30 alone; (A-b) xenograft tumors transplanted by CTPE-induced passage 30 BEAS-2B/THP-1 cells; (A-c) metastatic lymph node; (A-d) metastatic liver. (B) Quantitative comparison of the densitometry of c-Jun protein. The mean densitometry of cells with positive c-Jun staining in xenograft tumors transplanted by CTPE-induced passage 30 BEAS-2B/THP-1 cells higher than that in xenograft tumors from CTPE-treated BEAS-2B cells of passage 30 alone, in metastasis lymph nodes or metastasis livers. (* vs CTPE-treated BEAS-2B cells of passage 30, $p<0.05$; # vs metastatic lymph node, $p<0.05$; Δ vs metastatic liver, $p<0.05$)

transplanted by CTPE-induced passage 30 BEAS-2B/THP-1 cells was 0.350±0.011, which was higher than that in xenograft tumors from CTPE-treated BEAS-2B cells of passage 30 alone (0.276±0.008), in metastasis lymph nodes (0.267±0.014) and metastasis livers (0.303±0.012), and the differences were statistically significant ($P<0.05$) (Figure 4B).

Correlation analysis between c-Jun protein expression level and the number of tumor nests, microvasculars, metastasis

Correlation analysis showed that there was positive correlation between c-Jun expression levels and the number of microvasculars in CTPE-treated BEAS-2B cells of passage 30 group and CTPE-induced passage 30 BEAS-2B/THP-1 cells group (R=0.930, P=0.007), and there was also positive correlation between c-Jun expression levels and tumor metastasis in these two groups (R=0.905, P=0.013). But there was no correlation between c-Jun expression levels and the number of tumor nests (R=0.322, P=0.533).

Discussion

Macrophage was defined first by a bacteriologist, Ilya Mechnikov, more than a century ago. Monocytes are precursors of resident macrophages. Tumor-derived factors attract circulating monocytes into the tumor tissues where they differentiate into macrophages, which undergoes two distinct phenotypes, M1 macrophages (classically activated) and M2 macrophages (alternatively activated) (Biswas and Mantovani, 2010; Gordon and Martinez, 2010; Cui et al., 2013). It is mostly accepted that tumor associated macrophages (TAMs) have M2 phenotype and TAMs infiltration has been shown to correlate with poor prognosis in lung, breast, bladder cancer, papillary renal cell carcinoma and gliomas (Zhang et al., 2011; Wang et al., 2012; Ajili et al., 2013; Hutterer et al., 2013; Tang, 2013). Increasing evidence shows that TAMs have tumor-promotion function. In the pre-tumor stage, inflammatory cytokines and chemokines produced by TAMs can contribute to carcinogenesis transformation (Utrera-Barillas et al., 2010; Feng et al., 2012; Techasen et al., 2012). Infiltrating TAMs could produce a proliferation inducing ligand (APRIL) by direct stimulation with Helicobacter pylori (Hp) to promote the initiation of gastric lymphoma (Munari et al., 2011). In established tumors, TAMs secret factors that promote tumor growth, angiogenesis and metastatic spread of cancer cells, TAMs infiltrated in the invasive front are associated with improvement in hepatic metastasis in colon cancer (Zhou et al., 2010); and TAMs could provide a favorable microenvironment for non-small lung cancer invasion and progression (Wang et al., 2011). In our previous study, we observed that the expression levels of CD68+ TAMs were increased in adjacent lung tumor tissue from lung cancer patients and correlated with lung cancer progression and metastasis (Feng et al., 2012). In the present study, we evaluated the role of macrophages on the promotion of lung cancer progression and metastasis using a nude mice model and the mechanisms involved.

In this study, tumors derived from the mixture of CTPE-treated BEAS-2B cells at passage 30 and THP-1 cells were formed earlier than that from passage 30 CTPE-induced BEAS-2B cells alone (Figure 1A), and the average weight of tumors from two kinds of cell mixtures were larger than those from transformed BEAS-2B cells alone on the 30th day after transplantation (Figure 1B). Because THP-1 cells alone cannot form tumor (Kaler

et al., 2009; Kaler et al., 2010), these results indicate that macrophage-like THP-1 could promote lung cancer initiation and development.

Pathological alterations of xenografts were observed, which showed that the numbers of tumor nests and microvasculars in tumors from the mixture of CTPE-induced passage 30 BEAS-2B and THP-1 cells were increased apparently compared to those in tumor from the CTPE-treated BEAS-2B cells of passage 30 alone (Figure 2B, C). These results suggested that macrophage-like THP-1 cells facilitate angiogenesis in tumors to provide rapidly proliferating transformed-cells much more oxygen and nutrients. The proangiogenic role of TAMs has been characterized by previous studies that showed the correlation between TAMs infiltration and high number of vascular in many tumors, such as squamous cell carcinoma of the esophagus, prostate cancer and ameloblastoma (Koide et al., 2004; Halin et al., 2009; Guzman-Medrano et al., 2012), the main reason for this may be that TAMs produce many cytokines for angiogenesis: vascular endothelial growth factor (VEGF), platelet- derived growth factor (PDGF), TNF-α, transforming growth factor-β (TGF-β) (Bingle et al., 2006; Araujo et al., 2010; Shu et al., 2012), and several angiogenesis-modulating enzymes, such as matrix metalloproteinases (MMP) (Pollard, 2004; Kale et al., 2014). The fact that the specific proangiogenic program in tumor turns on indicated that tumor is in a condition of hypoxic, rendering tumor cells to angiogenesis. TAMs preferentially localize in tumor hypoxic regions, and hypoxic conditions promote hypoxia inducible factor-1 (HIF-1) and hypoxia inducible factor-1α (HIF-2) expression with subsequent overexpression of proangiogenic chemokines secreted from TAMs, such as CCL2, CXCL8, CXCL1, and CXCL13 (Balkwill, 2004). AP-1 has also been found to support angiogenesis, Laderoute and colleagues reported that AP-1 has close relationship with HIF-1α which promote macrophage-derived chemokines production (Laderoute, 2005). In the present study, a positive correlation between c-Jun expression level and the number of microvascular in tumor was also observed, which supported the role of proangeogenic function of AP-1.

Tumor metastasis process represents an important phase of tumor progression when tumor cells leave the primary sites and migrate to distant sites through blood or lymphatic vessels. In this present study, tumors metastasis to lymph nodes and liver after transplantation of CTPE-induced passage 30 BEAS-2B/THP-1 cells was detected (Figure 3), but no metastasis happened with injected CTPE-induced passage 30 BEAS-2B cells alone, which indicated that TAMs play a role in tumor metastasis. The association between TAMs and tumor metastasis has been examined by several studies (Condeelis and Pollard, 2006; DeNardo et al., 2008; Qing et al., 2012). Jeremy and colleagues (Chen et al., 2005) co-cultured THP-1 cells and lung caner cell line using Transwell system and found that lung cancer cell co-cultured with THP-1 showed strong potential of matrix deposition and invasion. And Wyckoff and colleagues (Wyckoff et al., 2004) demonstrated that interaction between macrophages and tumor cells facilitate their simultaneous migration and invasion. Recently, Chen and colleagues have provided a new mechanism for the metastasis-promoting function of macrophages through their adherence to tumor cells that provides survival signals to the tumor cells, by expressing vascular cell adhesion molecule-1 (VCAM-1) on macrophages (Chen et al., 2011). It has been recognized that TAMs promote tumor dissemination by producing related cytokines, coculture of tumor cells with macrophages enhances invasiveness if neoplastic cells undergo TNF-dependent MMP induction in macrophages (Hagemann et al., 2004). In addition, there was a positive correlation between c-Jun expression level and the number of tumor metastasis, which suggested AP-1 can modulate the extracellular matrix and affect invasion and metastasis of tumor cells (Eferl and Wagner, 2003). Fujisawa and colleagues demonstrated that tumors and metastatic lymph node expressed high levels of MMPs and high ERK/AP-1 activation in mouse model of human ovarian cancer (Fujisawa et al., 2012). The explanation for this might be that AP-1 could regulate macrophage to secrete tumor-promoting cytokines, such as TNF-α, which contribute to tumor metastasis (Choo et al., 2006).

In summary, macrophages not only facilitate tumorigenesis transformation of CTPE-induced BEAS-2B cells, but also promote tumor growth, angiogenesis and metastasis in a nude mice model. AP-1, a crucial transcription factor, may have a promoting role in the progression and metastasis of lung cancer.

Acknowledgements

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