Interleukin-1β activates focal adhesion kinase and Src to induce matrix metalloproteinase-9 production and invasion of MCF-7 breast cancer cells

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Abstract. Interleukin-1β (IL-1β) is a pleiotropic cytokine that is important in tumor progression and invasion. Matrix metalloproteinase-9 (MMP-9), which is a secreted matrix-degrading enzyme, is one of the key regulators of tumor invasion and metastasis. The current report indicated that IL-1β promotes MMP-9 production and cell invasion in non-metastatic MCF-7 breast cancer cells. IL-1β activated focal adhesion kinase (FAK) and proto-oncogene tyrosine-protein kinase Src (Src). Moreover, inhibiting the Src/FAK pathway reduced the IL-1β-induced production of MMP-9 and cell invasion. To investigate the functional role of FAK in MMP-9 production cell lines expressing mutant FAK in FAK knock-out mouse fibroblasts were generated. In wild-type FAK-expressing cells, MMP-9 production was induced by IL-1β stimulation. By contrast, IL-1β-induced MMP-9 production was abrogated in FAK knock-out, FAK Y397F, FAK Y925F, and kinase dead mutant-expressing cells. Therefore the results of the current study indicate that FAK and Src kinases are activated by IL-1β and play a critical role in MMP-9 production and tumor cell invasion.

Introduction

A number of epidemiological and clinical studies have presented evidence that chronic inflammation caused by microbial infection, as well as chemical irritants, significantly increases cancer risk (1,2). Obesity and post-partum involution are chronic inflammatory states in mammary glands, and are associated with increased breast cancer risk (3). Furthermore, non-steroidal anti-inflammatory drugs (NSAID), which are nonselective Prostaglandin G/H synthase 2, or cyclooxygenase-2 (COX-2) inhibitors, significantly reduce the risk of mammary carcinogenesis, recurrence, and motility of breast cancer (4). In the tumor microenvironment, various inflammatory cells, such as tumor-associated macrophages, are recruited to form a pro-tumor inflammatory environment. Inflammatory cells produce a variety of mediators, including growth factors, chemokines and cytokines, which induce tumor growth, invasion, and angiogenesis (5,6).

Proinflammatory cytokines are a major determinant for the invasiveness of tumor cells. Among these cytokines, interleukin-1β (IL-1β) is abundant in tumor tissues and stimulates tumor growth, invasion, carcinogenesis and host-tumor association (7). IL-1β-knockout mice are resistant to the development of chemically induced tumors (8), and exhibit suppressed tumor invasion and angiogenesis (9,10). On the other hand, stomach-specific IL-1β overexpression induces gastric inflammation and cancer (11). These results identify IL-1β as one of the essential components mediating inflammation-associated tumor progression. IL-1β stimulation of tumor cells activates multiple signaling pathways involving protein kinase B, mitogen activated protein (MAP) kinase, and nuclear factor-kB (12). Activation of these signaling molecules is required for IL-1β-mediated production of matrix metalloproteinase (MMP)-9, a matrix degrading enzyme that is regarded as a critical regulator in IL-1β-induced tumor invasion (13-15).

A number of studies have investigated the association between MMP expression and the prognosis of breast cancer patients. Most recently, a meta-analysis by Ren et al (16) reported that MMP-9 overexpression in serum was associated with poor patient prognosis in breast cancer.

Proto-oncogene tyrosine-protein kinase Src (Src) is a non-receptor tyrosine kinase that is comprised of SH3, SH2, and kinase domains. Extracellular stimuli including cytokines, growth factors and integrin engagement, activate Src, which in turn, phosphorylates various target proteins to regulate cell proliferation, differentiation, and migration (17,18). Among these target proteins, focal adhesion kinase 1 (FAK) is essential for the regulation of signal transduction, cell adhesion and migration carried out by Src (19). FAK is composed of an N-terminal FERM domain, a central kinase domain, and a C-terminal focal adhesion targeting domain. Additionally, FAK localizes to the site of cell-extracellular matrix contact (20). There are six major tyrosine phosphorylation sites in FAK, and two of them, the Tyr397 and Tyr925 sites, are important for FAK-dependent signaling (21). Src interacts with the phosphorylated Tyr397 of FAK and phosphorylates...
Ytr925, which in turn associates with signaling molecules such as growth factor receptor-bound protein 2 (Grb2) to induce activation of the Ras-dependent/MAP kinase pathway (19,22).

It has previously been reported by this group that the enhancement of cell invasion caused by nitric oxide stimulation is mediated by Src and FAK kinase activation in MCF-7 breast cancer cells (23). To expand on these findings, the current study aimed to examine the role Src and FAK serve in the IL-1β-mediated cell invasion of MCF-7 cells, and identify whether Src and FAK kinase are involved in MMP-9 production and cell invasion.

Materials and methods

Antibodies, cytokines and chemicals. Recombinant murine IL-1b and recombinant human IL-1b were purchased from PeproTech EC (London, UK). The PP2 kinase inhibitor (PP2) was purchased from EMD Millipore (Billerica, MA, USA). Anti-FAK (cat. no. sc-154; 1:1,000), anti-phospho-Erk (cat. no. sc-7383; 1:1,000) and anti-Erk2 (cat. no. sc-558; 1:1,000) antibodies were all obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA); anti-phosphotyrosine antibody (pTyr20; cat. no. 610012; 1:1,000) was purchased from BD Transduction Laboratories™ (BD Biosciences, Franklin Lakes, NJ, USA); anti-phospho-FAK antibody (pTyr397; cat. no. 44624G; 1:500) was purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA); and anti-phospho-Src (pTyr416; cat. no. 6943; 1:1,000) and anti-phospho-FAK (pTyr925; cat. no. 3284; 1:1,000) antibodies were both obtained from Cell Signaling Technology (Danvers, MA, USA).

Cell culture, plasmid construction, and transfection. The human breast cancer cell line, MCF-7, was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan), and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Biowest Europe, Nuaillé, France) and 5 mg/ml human insulin (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). Subsequently, knockout of FAK was performed to establish homozygous null FAK knockout fibroblast cells, following a previously documented procedure by Ilić et al (24). To establish FAK-wild-type (FAK-Wt), FAK-kinase dead (KD), FAK-Y937F, and FAK-Y925F cells, wild-type and mutant FAKs were cloned into a pBabePuro vector and transfected into FAK-Ko cells.

Assay of gelatin-degrading MMPs by zymography. The activity of MMPs in the conditioned media was assayed by zymography as described previously (21). Briefly, cells were incubated in serum-free medium for 6 h followed by stimulation with or without IL-1b for 16 h. Conditioned media were collected, clarified by centrifugation, and subjected to electrophoresis with sodium dodecyl sulphate-polyacrylamide gels. Gels were washed and incubated with reaction buffer (50 mM Tris-HCl, pH 7.4, 0.02% Na2EDTA, 10 mM CaCl2) for 16 h at 37°C, stained with Coomassie brilliant blue, and subsequently destained.

FAK siRNA and transfection. The sequence of FAK siRNA is 5'-CCACCUGGGCCAGUAUAAUTT-3', and the sequence for luciferase siRNA is 5'-CUUACGCGAGACUUCGATT-3'. Cells were transfected with 20 nM of each siRNA using Lipofectamine™ RNAiMax (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturers' protocol.

Invasion assay. MCF-7 cells were assayed for their invasiveness by a modified Boyden chamber method as described previously (21). Cells were serum-starved for 6 h and pretreated with varying concentrations of IL-1b (0, 0.5, 1, 3, 5, 10 nM) for 12 h. Cells were subsequently resuspended in serum-free DMEM and seeded onto Matrigel-coated filters with or without IL-1b. Following incubation for 7 h, cells that had invaded the lower surface of the filter were fixed, stained, and quantified by counting three randomly selected fields under the microscope.

The mean ± standard deviation (SD) of three independent experiments was calculated. To examine cell invasion in the absence of FAK expression, cells were incubated with the indicated siRNAs for 30 h, serum-starved for 6 h, and treated with IL-1b for 12 h before undergoing an invasion assay. To investigate cell invasion with PP2, cells were serum-starved for 6 h, treated with 10 µM PP2 for 1 h, stimulated with IL-1b for 12 h and subjected to an invasion assay.

Statistical analysis. Values are expressed as the mean ± SD of three independent experiments. Comparisons between groups were performed using an unpaired Student's t-test. Statistical analysis was performed using GraphPad Prism software (version 7.0; GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 were considered to indicate a statistically significant difference.

Results

IL-1b induces invasion and MMP-9 production in MCF-7 cells. The effect of IL-1b stimulation on the invasiveness of non-metastatic MCF-7 human breast cancer cells in vitro was examined. The level of invasion by MCF-7 cells was low in the absence of IL-1b stimulation. However, increasing the concentration of IL-1b increased the invasiveness of MCF-7 cells in a dose-dependent manner (Fig. 1A). MMPs are matrix-degrading enzymes essential for tumor cell invasion. Previous studies have demonstrated that IL-1b activates MMP-9 secretion (14). Therefore, the production of MMP-9 in MCF-7 cells following IL-1b stimulation was examined. MCF-7 cells were serum-starved, treated with the indicated concentrations of human IL-1b for 16 h prior to gelatin zymography. Although MMP-9 production was undetectable in MCF-7 cells in the absence of IL-1b, IL-1b stimulation increased MMP-9 expression in a dose-dependent manner (Fig. 1B). By contrast, the production of MMP-2 was limited even at higher doses of IL-1b (Fig. 1B).

FAK and Src are activated by IL-1b stimulation in MCF-7 cells. It was previously demonstrated by this group that FAK activation is responsible for the increased production of MMP-9 by fibronectin and tumor necrosis factor α (25,26). Thus, the current study investigated whether FAK was activated by IL-1b stimulation. To measure tyrosine phosphorylation of FAK, MCF-7 cells were serum-starved, treated with 3 nM IL-1b, and lysed by immunoprecipitate FAK, and
western blotting was subsequently carried out. Treatment of MCF-7 cells with IL-1β increased FAK tyrosine phosphorylation in a time-dependent manner (Fig. 2A). Furthermore, the phosphorylation of Tyr397 and Tyr925 in FAK was evaluated. Tyr397 is auto-phosphorylated when the kinase is activated, and Tyr925, which functions as a binding site for Grb2 to activate the Ras/ERK pathway, is phosphorylated by Src. The phosphorylation of these tyrosine residues was induced by IL-1β stimulation (Fig. 2A). Src regulates the phosphorylation of Tyr925, therefore its activation following IL-1β stimulation was assessed. The phosphorylation of Tyr416, which regulates the catalytic activity of Src, was increased by IL-1β stimulation (Fig. 2B).

**FAK and Src are required for IL-1β-induced MMP-9 production and invasion in MCF-7 cells.** The effect of silencing FAK on the production of MMP-9 in MCF-7 cells was investigated. MCF-7 cells were transfected with FAK siRNA, and the production of MMP-9 after IL-1β treatment was examined by zymography. Knockdown of FAK expression by siRNA reduced the production of MMP-9 by IL-1β (Fig. 3A). The role of FAK in the IL-1β-induced invasion of MCF-7 cells was then examined using a modified Boyden chamber. MCF-7 cells were transfected with either a luciferase or FAK siRNA, and after 30 h, they were starved and stimulated with IL-1β. Cells were then loaded onto the upper chamber and incubated with or without IL-1β. Following 7 h, cells that had invaded the lower surface of the chamber were fixed, stained and quantified by counting. The results demonstrated that FAK siRNA significantly suppressed cell invasion (Fig. 3B).

To determine whether Src is required for IL-1β-mediated production of MMP-9, the Src inhibitor PP2 was used. MCF-7 cells were stimulated with IL-1β with or without PP2, MMP-9 production was examined, and it was observed that PP2 treatment markedly suppressed MMP-9 production (Fig. 3C).

A previous study had demonstrated that the activation of extracellular signal-related kinases (Erk) was required for MMP-9 production (12). The current study demonstrated that stimulation of MCF-7 cells with IL-1β led activated Erk, which in turn was inhibited by PP2 treatment. In addition, the combined treatment of PP2 and FAK siRNA reduced the phosphorylation of Erk, which is mediated by IL-1β (Fig. 3C). This indicates that the Src/FAK pathway is crucial for the activation of Erk by IL-1β, and production of MMP-9.

The requirement of Src for IL-1β-induced invasion of MCF-7 cells was examined using a modified Boyden chamber.
IL-1β-induced cell invasion was suppressed by PP2 treatment, and the combined treatment of PP2 and FAK siRNA significantly reduced cell invasion (P=0.0014; Fig. 3D). Therefore, FAK and Src are both required for IL-1β-mediated MMP-9 production and cell invasion.

**IL-1β-mediated MMP-9 production is dependent on the activation of FAK.** To confirm the role of FAK in IL-1β-induced MMP-9 production, FAK-Ko cells and FAK-Wt cells (generated by transfecting FAK-Ko cells with wild-type FAK) were used. The cells were serum-starved and stimulated with varying concentrations of IL-1β (0, 1, 3, 5, and 10 nM) before undergoing gelatin zymography. The results demonstrated that IL-1β stimulation increased the production of MMP-9 by FAK-Wt cells (Fig. 4A). By contrast, an increase of MMP-2 production stimulated by IL-1β was limited in FAK-Wt cells. FAK-Ko cells responded weakly to IL-1β treatment, and the production of MMP-9 was markedly lower than untreated FAK-Wt cells (Fig. 4A). These results indicate that FAK is crucial for IL-1β-mediated MMP-9 production.

The effect of IL-1β stimulation in FAK-Wt cells was subsequently examined, to identify whether FAK and Src were activated. FAK-Wt cells were serum-starved, stimulated with 3 nM IL-1β, and the expression of phosphorylated FAK and Src was examined by western blot analysis. This demonstrated that IL-1β enhanced the tyrosine phosphorylation of FAK and Src in FAK-Wt cells (Fig. 4B and C).

Finally, to investigate the functional role of FAK for MMP-9 production, cell lines expressing mutant FAK were established. FAK-Y397F and FAK-Y925F are mutant FAKs in which Tyr397 and Tyr925 are replaced with phenylalanine, respectively. FAK-KD lacks kinase activity due to replacement of lysine 454 with arginine. Cell lines expressing these mutants did not increase MMP-9 expression in the presence of IL-1β (Fig. 4D). These data suggest a critical role of FAK activity in IL-1β-dependent MMP-9 production.

**Discussion**

IL-1β is abundant at tumor sites, and it induces the expression of various genes to facilitate malignant cell invasion (7). Src and FAK are critical regulators that control cell attachment, migration and signal transduction which are associated with invasion and metastasis (27-29). The present report investigated...
whether Src and FAK are required for IL-1b-mediated MMP-9 production and cell invasion. Using FAK-Ko mouse fibroblasts and FAK siRNA-transfected MCF-7 cells, it was demonstrated that FAK is essential for IL-1b-induced MMP-9 production and cell invasion. Furthermore, suppression of Src activity by a chemical inhibitor decreased IL-1b-induced MMP-9 production and cell invasion, indicating that Src activation is required to induce MMP-9 via stimulation of IL-1b. These results demonstrate that activation of the Src/FAK pathway is crucial for cell invasion following IL-1b stimulation.

MMPs are major proteolytic enzymes required for tumor invasion and angiogenesis. Among MMPs, MMP-9 secretion is observed in different types of cancer, and its production is regulated by extracellular stimuli, such as growth factors and cytokines (30). Although the signaling pathways required for MMP-9 production differ depending on the extracellular stimuli, Erk activation seems to be essential (14,31). In the current study, it was observed that inhibiting the Src/FAK pathway significantly reduced the activation of Erk by IL-1b. It had previously been demonstrated that phosphorylating Tyr397 and Tyr925 induces the activation of the Ras/Erk pathway (19). In the present study, cells expressing mutant FAK, which consisted of Tyr397 and Tyr925 substituted to alanine, exhibited only a limited increase of MMP-9 production following IL-1b stimulation. These results suggest that activation of the Src/FAK pathway is required for the Erk activation by IL-1b, which in turn promotes MMP-9 production.

Previous studies have indicated that Src and FAK are related to cancer progression and invasion (32-34). Elevated Src expression has been observed in different types of cancer, including colon, breast, pancreatic and gastric cancer (27). In addition, genetic analysis has revealed an activating mutation in the C-terminus of Src in a subset of metastatic colon cancers (35). Overexpression of FAK has been observed in various types of invasive cancer, and FAK activity in malignant cells is correlated with invasiveness (36). Both Src and FAK are critical for the assembly of focal adhesions and cytoskeleton to induce tumor cell invasion (37). In addition to these critical functions, the current study indicated that Src and FAK are required for IL-1b-induced cell invasion and MMP-9 production. Taken together, these results indicate that the Src/FAK pathway plays a pivotal role in inflammation-mediated tumor cell invasion.

IL-1b promotes MMP-9 production and cell invasion in non-metastatic MCF-7 breast cancer cells. Src and FAK activation are important for MMP-9 production and cell invasion by IL-1b stimulation and IL-1b-induced Erk activation is dependent on the activation of the Src/FAK pathway. Systemic treatment of mice with the IL-1 receptor antagonist (IL-1Ra), a physiological inhibitor of IL-1 signaling, inhibits tumor growth and metastasis, indicating that targeting IL-1b signaling is a promising therapy for cancer (9). Both Src and FAK have been extensively studied over the last decade. However, therapeutically targeting Src and FAK has only generated substantial interest recently (38).

In conclusion, the results of the present study suggest that the inhibition of the Src/FAK pathway is an effective treatment for inflammation-associated tumor growth and invasion.

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References