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Luteolin inhibits recruitment of monocytes and migration of Lewis lung carcinoma cells by suppressing chemokine (C–C motif) ligand 2 expression in tumor-associated macrophage

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ABSTRACT

Tumor-associated macrophages (TAMs) play pivotal roles in the progression of cancer. In order to investigate a novel candidate that inhibits the tumor-supporting M2-like phenotype of TAMs, a murine macrophage cell line RAW 264.7 cells were treated with interleukin (IL)-4. Luteolin inhibited phosphorylation of signal transducer and activator of transcription 6 (STAT6), a main downstream signal of IL-4, and reduced the expression of the M2-associated genes. In addition, Luminex multiplex analysis for secreted cytokines revealed that IL-4–enhanced secretion of chemokine (C–C motif) ligand 2 (CCL2) was reduced by luteolin treatment. IL-4–stimulated migration of monocyte, THP-1 cells, was inhibited by luteolin treatment and recovered by recombinant CCL2 supplement. Moreover, luteolin decreased migration of Lewis lung carcinoma cells in a CCL2–dependent manner. Given the important role of the TAM phenotype in the tumor microenvironment, inhibitory effect of luteolin on the monocyte recruitment and cancer migration via suppression of the TAM-secreted CCL2 may suggest a novel therapeutic approach to treat malignant tumors.

1. Introduction

Cancer cells recruit and corrupt surrounding normal cells to serve as contributors to tumor-progression [1]. Therefore, it is commonly accepted that cancer cannot be understood simply by determining the characteristics of cancer cells alone, but the tumor microenvironment that includes surrounding normal cells should also be considered [1]. Macrophages are the major components in tumor microenvironment, and display functional and anatomical diversity depending on the surrounding environment [2]. Macrophages originate from blood monocytes, differentiate into distinct macrophages, and are schematically identified by classically activated (M1) or alternatively activated (M2) phenotypes [1]. The tumor-associated macrophage (TAM) generally resembles the M2 phenotype, and is polarized to promote tumor growth, migration, invasion, and metastasis [3]. In addition, an abundance of TAMs is related to poor prognosis of cancer and poor response to chemotherapy or radiation [4]. Given this background, regulation of the TAM phenotype has been considered an important target of cancer treatment.

Previous researches have shown that several drugs are effective for cancer suppression via macrophage regulation [5]. For example, clodronate, a hydrophilic phosphonate that is used as an anti–osteoporotic drug, can induce the apoptosis of macrophages. Chemical deletion of TAM by using liposomal clodronate prohibited growth of osteosarcoma [6]. Tyrosine kinase inhibitors are also promising candidates for TAM-directed therapy because macrophage polarization is regulated by tyrosine kinases. For example, sorafenib, a multi-tyrosine kinase inhibitor, altered macrophage polarization and consequently reduced cancer growth [7]. Additionally, sorafenib triggered pro-inflammatory activity of TAM and subsequently induced antitumor NK cell responses [7]. However, chemical elimination of macrophages or broad inhibition of tyrosine kinases can induce unexpected side–effects [8]. Therefore, there is a need to find novel candidates that specifically inhibit the M2 phenotype of macrophages.

Luteolin, 3′,4′,5,7-tetrahydroxyflavone, is a common flavonoid derived from various herbal plants [9]. Anti–tumor activities of

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luteolin mediated by several mechanisms, including inhibition of cancer cell proliferation, induction of apoptosis, anti–angiogenesis, and anti–metastasis, were reviewed [10]. However, the function of luteolin on TAM polarization has not yet been investigated. Herein, we show that luteolin inhibits interleukin (IL)-4–induced phosphorylation of signal transducer and activator of transcription 6 (STAT6) and the TAM phenotype. Consequently, this ameliorates the recruitment of monocytes and the migration of lung cancer cells. This effect is mediated by the reduction of IL-4–stimulated chemokine (C–C motif) ligand 2 (CCL2) secretion from macrophages. Given the importance of TAM in tumor progression, luteolin might be a novel candidate for regulating the tumor microenvironment.

2. Materials and methods

2.1. Materials

Luteolin was purchased from Sigma–Aldrich (St. Louis, MO, USA), and the purity was over than 98% certified by supplier. Anti-pSTAT6 (sc-11762-R) and anti-STAT6 (sc-981) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-actin (A2066) antibody was purchased from Sigma–Aldrich. Anti-arginase-1 (ab60176) antibody was from Abcam (Cambridge, UK). Dimethyl sulfoxide (DMSO) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich. Cell proliferation kit II (XTT) was supplied from Roche Life Science (Penzverg, Germany). Recombinant human IL-4 and CCL2 were purchased from Peprotech (Rocky Hill, NJ, USA). Lipopolysaccharide (LPS) was from Enzo life sciences (Farmingdale, NY, USA).

2.2. Cell culture

The murine macrophage cell line, RAW 264.7 cell, the human acute monocytic leukemia cell line, THP-1 cell, and the murine Lewis lung carcinoma cell line, LLC cell, were obtained from American Type Culture Collection (ATCC; Rockville, MD, USA). RAW 264.7 cells and LLC cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) and THP-1 cells were grown in Roswell Park Memorial Institute medium (RPMI-1640; Thermo Fisher Scientific) containing L-glutamine (200 mg/L; Thermo Fisher Scientific). Culture media were supplemented with 10% (v/v) heat–inactivated fetal bovine serum (FBS; Sigma–Aldrich), and 100 U/mL penicillin and 100 μg/mL streptomycin (Thermo Fisher Scientific) at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. Cell viability assays

Cells were treated with the indicated concentration of luteolin for 24 h. For estimating the viability of adhesive cells, including RAW 264.7 cells and LLC cells, MTT was added at a concentration of 50 mg/mL to the CM and then incubated for 4 h. The formazan salt was dissolved with DMSO and optical density was measured at 590 nm using a SpectraMax M2 (Molecular devices, Sunnyvale, CA, USA). The viability of suspension cell cultures, such as THP-1 monocytes, was estimated using XTT. Briefly, 50 μL XTT labeling mixture was added to cell suspensions (5×10^4 cells/well in 96-well plates) and incubated for 4 h. The wavelength used to measure the absorbance of the formazan product was 450 nm.

2.4. Western blot analysis

Cells were lysed in RIPA buffer (1% NP-40, 1M HEPES (pH7.45) buffer solution, 150 mM NaCl, 2 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 5 mM sodium fluoride). The cell lysates were separated by electrophoresis on 8–10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting. The membranes were blocked with 5% skimmed milk for 1 h and incubated with primary antibody overnight at 4 °C. The blots were incubated with the appropriate secondary antibody for 1 h after washing with TBS-T (50 mM Tris/pH 7.5, containing 0.15 M NaCl, and 0.05% Tween-20). The blots were developed in ECL mixture (Thermo Fisher Scientific).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from RAW 264.7 cells using the GeneJET RNA Purification Kit (Thermo Fisher Scientific). Synthesis of cDNA was carried out using AccuPower RT-PreMix (Bioneer, Daejeon, Korea) and amplification of target genes were performed using AccuPower® PCR PreMix (Bioneer). The sequences of the primers used for the PCR assays were as follows: 5′–TAGAGCTCAACAGGCAGGCTGCAAGGAAT–3′ and 3′–TAGAGCTCCTGTCCAGCTCCTCTCCACCG–5′ for *arginase-1*, 5′–GCGCTGCGTGGACGCTCTAA–3′ and 3’–CCAGAGCCATCCGTCCGAGC–5′ for *MRC 1*, 5′–CTGCAGCACTTGGATCAGGAACC–3′ and 3′–GGGAGTAGCCTGTGTGCACCTGGAA–5′ for *iNOS*, 5′–GCCCATCCTCTGTGACTCAT–3′ and 3′–AGGCCACAGGTATTTTGTCG–5′ for *IL-1β*, 5’–AGGTCCCTGTCATGCTTCTG–3′ and 3′–TCTGGACCCATTCCTTCTTG–5′ for *CCL2* and 5′–AACTTTGGCATTGTGGAAGG–3′ and 3′–ACACATTGGGGGTAGGAACA–5′ for *GAPDH*.

2.6. Measurement of secretory cytokines

RAW 264.7 cells were treated with luteolin and IL-4 and incubated for 24 h. The supernatants of cells were collected and stored at −80 °C before use for cytokine measurements. Cytokines secreted from differently treated RAW 264.7 cells were measured by a commercial service using the Luminex multiplexing system (Koma Biotech, Seoul, Korea). The concentrations of secreted cytokines were expressed as mean ± SD.

2.7. Cell migration assays

In vitro cell migration assays were performed using 24-well transwell inserts (8 μM). In order to determine the migration changes of THP-1 cells, RAW 264.7 cells (1×10^5 cells/well) were placed in the lower chambers of the transwells and then treated with luteolin for 1 h before IL-4 stimulation. After 9 h, rh-CCL2 (60 ng/mL) was added and THP-1 monocytes were added to the upper chamber. Cells were incubated for 16 h. THP-1 cells that migrated into lower chamber were collected, centrifuged, and then counted by using a hemocytometer. For estimating LLC cell migration, RAW 264.7 cells (1×10^5 cells/well) were added to the lower chamber and treated with luteolin and subsequently, IL-4, after a 1 h interval. After 9 h, rh-CCL2 (60 ng/mL) was added to reverse the effect of luteolin. LLC cells (5×10^4 cells/well) were seeded in the upper chambers of the transwells and incubated for 24 h. LLC cells that migrated to the lower part of the membrane were stained using hematoxylin and eosin (Sigma–Aldrich) and counted.

2.8. Statistical analysis

Values for cell viability, cytokine levels, and migration rates

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