Biochemical Pharmacology 85 (2013) 898–912

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Morin inhibits STAT3 tyrosine 705 phosphorylation in tumor cells through activation of protein tyrosine phosphatase SHP1

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ARTICLE INFO

Article history:
Received 7 November 2012
Accepted 20 December 2012
Available online 29 December 2012

Keywords:
Apoptosis
Chemosensitization
Flavonol
SHP-1
STAT3

ABSTRACT

The major goal of cancer drug discovery is to find an agent that is safe and affordable, yet effective against cancer. Here we show that morin (3,5,7,2′,4′-pentahydroxyflavone) has potential against cancer cells through suppression of the signal transducer and activator of transcription 3 (STAT3) pathway, which is closely linked to the transformation, survival, proliferation, and metastasis of cancer. We found that morin completely suppressed inducible and constitutively activated STAT3 and blocked the nuclear translocation of STAT3 and its DNA binding in multiple myeloma and head and neck squamous carcinoma cells. Morin inhibited activated Src, JAK-1, and JAK-2, all of which are linked to STAT3 activation, while up-regulating a protein inhibitor of activated STAT3, PIAS3. Pervanadate reversed the effects of morin on STAT3 phosphorylation, indicating the role of a protein tyrosine phosphatase. Furthermore, morin induced SHP1 expression at both the mRNA and protein levels, and silencing of SHP1 abrogated the effect of morin on STAT3 phosphorylation, indicating that morin mediates its effects on STAT3 through SHP1. Suppression of STAT3 correlated with the down-regulation of various gene products linked to tumor survival, proliferation, and angiogenesis and led to sensitization of tumor cells to thalidomide and bortezomib. Comparing the activities of morin with those of four structurally related flavonols demonstrated the importance of hydroxyl groups in the B ring in inhibiting STAT3 activation. These findings suggest that morin suppresses the STAT3 pathway, leading to the down-regulation of STAT3-dependent gene expression and chemosensitization of tumor cells.

1. Introduction

Effective anticancer agents that are safe, nontoxic, and affordable are urgently needed. Although a number of agents derived from fruits, vegetables, and nuts have been linked to the prevention and treatment of cancer, the ways in which these agents prevent disease remain largely unknown [1]. One such agent that may have potential against cancer is morin (3,5,7,2′,4′-pentahydroxyflavone), which was originally isolated from members of the *Moraceae* family such as mulberry figs and old fustic (*Chlorophora tinctoria*). Previous studies have found that morin suppresses the proliferation of a wide variety of tumor cells, including oral squamous cell carcinoma [2] and leukemia [3] cells, and inhibits the growth of COLO205 colorectal cancer cells in nude mice [4]. Other studies have demonstrated that morin reduces the incidence of lipopolysaccharide-induced septic shock [5] and suppresses the phorbol ester-induced transformation of hepatocytes [6]. Morin has also been found to exert chemopreventive effects in a model of dimethylhydrazine-induced colon carcinogenesis [7], induce p21, activate caspases [4], suppress AKT activation [2], and abolish peroxisome proliferator-activated receptor activity [8]. The ways in which morin exerts these activities is not fully understood; however, morin has been shown to modulate the nuclear factor (NF)-κB transcription factor in leukemic [3] and hepatocellular [9] carcinoma.

A number of studies have suggested that signal transducer and activator of transcription 3 (STAT3) plays a major role in the pathogenesis of various cancers. First, constitutively active STAT3 has been reported in multiple myeloma and various other cancers [10,11]. Second, constitutive STAT3 has been reported to participate in tumorigenesis by regulating the expression of genes involved in tumor cell proliferation (*e.g.*, c-myc, cyclin D1), survival (*e.g.*, Mcl-1, Bcl-xL, survivin), invasion (*e.g.*, matrix metalloproteinase-9), and angiogenesis (*e.g.*, VEGF) [10]. Third, STAT3 is activated in response to various growth factors including IL-6 and EGF [12]. Fourth, STAT3 activation has been linked with chemoresistance and radioresistance [13]. Fifth, STAT3 activation has been correlated with decreased survival in patients with multiple myeloma [10] and other cancers. These findings suggest

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http://dx.doi.org/10.1016/j.bcp.2012.12.018

that STAT3 is an attractive therapeutic target for the development of anticancer agents.

STAT3 has been shown to be a client protein of Hsp90 that is degraded by the ubiquitin–proteasome pathway [14,15]. In normal tissues, STAT3 is widely expressed as inactive monomers in the cytoplasm. Upon activation, STAT3 undergoes phosphorylation at tyrosine 705 (Tyr^{705}). This phosphorylation allows the STAT3 molecules to form homodimers, which enter the nucleus and regulate the transcription of genes involved in tumorigenesis. The phosphorylation of STAT3 at Tyr^{705} is mediated through the activation of non-receptor protein tyrosine kinases, including JAK-1, JAK-2, and Src kinase [10]. STAT3 also undergoes phosphorylation at Ser^{727} through mediation of kinases of the mitogen-activated protein kinase family [16]. Whereas these kinases positively regulate the STAT3 pathway, SH2-containing tyrosine phosphatases (SHP1 and SHP2) negatively regulate STAT3 activation by catalyzing tyrosine dephosphorylation of JAKs and other cellular proteins [17]. In addition, STAT3 is also negatively regulated by a group of other signaling proteins, such as protein inhibitors of activated STAT (PIAS) [18]. Thus, agents that can suppress STAT3 activation have great potential for therapeutic intervention.

Given STAT3's critical role in tumor cell development and morin's potential to modulate molecules involved in tumorigenesis, we postulated that morin exerts its therapeutic effects by modulating the STAT3 cell-signaling pathway. Therefore, we investigated the potential of morin to inhibit STAT3 activation and delineated the molecular mechanism. We focused mainly on multiple myeloma because it is the second most common hematological cancer in the United States, and the cure for this disease is elusive [19]. We showed that morin suppressed the activation of the STAT3 pathway in multiple myeloma cells. Morin up-regulated SHP1 and PIAS3 and suppressed the activation of various kinases (JAK-1, JAK-2, and Src). Morin-induced suppression of STAT3 activation down-regulated STAT3-regulated gene products and sensitized multiple myeloma cells to chemotherapeutic agents.

2. Materials and methods

2.1. Reagents

The flavonols galangin, kaempferol, quercetin, morin, and myricetin were purchased from Sigma Chemicals (St. Louis, MO). A 50 mM solution of the flavonols was prepared in dimethyl sulfoxide and then diluted as needed in cell culture medium. Hoechst 33342 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma–Aldrich (St. Louis, MO). RPMI 1640, FBS, penicillin–streptomycin, and the kit for the live/dead assay were obtained from Invitrogen (Grand Island, NY). Antibodies against STAT3, pSTAT3 (Tyr^{705}, Ser^{727}), Hsp90, Fas, STAT1, pSTAT1 (Tyr^{701}), Bcl-2, Bcl-xL, Mcl-1, c-IAP-2, cytochrome *c*, c-Myc, SHP1, SHP2, cyclin D1, procaspase-3, procaspase-8, procaspase-9, JAK-2, ERK2, p-ERK1/2 (Thr^{177}/Thr^{160}), poly (ADP-ribose) polymerase (PARP), PIAS3, and PIAS1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to pSrc (Tyr^{416}), Src, pJAK-1 ($Tyr^{1022/1023}$), pJAK-2, and JAK-1 were obtained from Cell Signaling Technology (Beverly, MA). Anti-X-linked inhibitor of apoptosis protein (XIAP) was purchased from BD Biosciences (San Jose, CA). Survivin antibody was purchased from R and D Systems (Minneapolis, MN). VEGF antibody was purchased from NeoMarkers (Fremont, CA). The cellular caspase-8 (FLICE)-like inhibitory protein (cFLIP) antibody was obtained from Imgenex (San Diego, CA). The siRNA for SHP1 and the scrambled control were obtained from Ambion (Austin, TX). Bacteria-derived recombinant human IL-6 was obtained from Novartis Pharmaceuticals (East Hanover, NJ). Bortezomib (PS-341) was obtained from Millennium (Cambridge, MA), and thalidomide was obtained from Tocris Cookson (Ellisville, MO). GST-JAK-2 substrate containing a peptide fragment with a sequence of PQDKEYYKVKE derived from the autophosphorylation sites of human JAK-2 tagged with a GST fusion protein, was provided by Dr. Zhizhuang Joe Zhao (Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, OK).

2.2. Cell lines

Human multiple myeloma cells (U-266, RPMI-8226, and MM.1S), breast cancer cells (MCF-7), and head and neck cancer cells (SCC-4) were obtained from the American Type Culture Collection (Manassas, VA). MCF-10A cells (human nontumor breast cells) were supplied by Dr. Kapil Mehta of our institute. U-266 and RPMI-8226 are plasmacytomas of B-cell origin. Whereas U-266 cells produce IL-6 and are resistant to glucocorticoids, RPMI-8226 cells produce Ig L chains but not H chain or IL-6 [20,21]. MM.1S is a glucocorticoid-sensitive cell line established from the peripheral blood of a patient with IgA myeloma. MCF-7 is a mammary gland tumorigenic epithelial cell line, whereas MCF-10A is a nontumorigenic epithelial cell line. SCC-4 cells, known to form colonies in semisolid medium and to express constitutively active STAT3, have been used extensively for delivery of siRNA. All of these cell lines have been used extensively for elucidating the mechanisms of action of anticancer agents. U-266, RPMI-8226, and MM.1S cells were cultured in RPMI 1640 medium containing 10% FBS. MCF-7 cells were cultured in DMEM containing 10% FBS. SCC-4 cells were cultured in DMEM containing 10% FBS, nonessential amino acids, pyruvate, glutamine, and vitamins. All media were supplemented with penicillin (100 units/mL) and streptomycin (100 μg/mL). MCF-10A cells were cultured in DMEM/F12 with 15 mM HEPES buffer, 5% horse serum, 10 μg/mL insulin, 20 ng/mL EGF, 100 ng/mL choleratoxin, 0.5 μg/mL hydrocortisone and penicillin–streptomycin. Cells were maintained at 37 °C in an atmosphere of 5% CO_2 and 95% air.

2.3. Extraction of cytoplasmic and nuclear extract

To examine the relative proportion of STAT3 in the cytoplasm and nucleus, cytoplasmic and nuclear fractions were prepared. In brief, control and treated cells were dissolved in a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA (pH 8.0), 0.1 mM EGTA (pH 7.0), 1 mM DTT, 0.5 mM PMSF, 2 μg/mL leupeptin, 2 μg/mL aprotonin, and 0.5 mg/mL benzamidine. After 20 min, NP-40 (0.25%) was added, and cell lysate was vortexed and centrifuged at 10,000 rpm for 1 min. The cytoplasmic fraction was taken and pellet was re-dissolved in a buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA (pH 8.0), 1 mM EGTA (pH 7.0), 1 mM DTT, 1 mM PMSF, 2 μg/mL leupeptin, 2 μg/mL aprotonin, and 0.5 mg/mL benzamidine. After 30 min, the extract was centrifuged at 12,000 rpm for 12 min, and nuclear extract was taken. The protein content in the nuclear and cytoplasmic fractions was determined by the Bradford method.

2.4. Electrophoretic mobility shift assay for STAT3–DNA binding

To analyze STAT3–DNA binding, we performed the electrophoretic mobility shift assay (EMSA) by using a ^{32}P-labeled high-affinity sis-inducible element probe (5′-CTTCATTTCCCGTAAATCCC-TAAAGCT-3′ and 5′-AGCTTTAGGGATTTA CGGGAAATGA-3′) [22]. In brief, nuclear extracts prepared from the control and morin-treated cells were incubated with the probe at 37 °C. The resultant DNA–protein complex was separated on 5.5% native polyacrylamide