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Oleanolic acid inhibits macrophage differentiation into the M2 phenotype and glioblastoma cell proliferation by suppressing the activation of STAT3

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Abstract. Tumor-associated macrophages (TAMs) polarized to the M2 phenotype promote tumor cell proliferation and are associated with a poor prognosis in patients with high grade glioma. We previously revealed that corosolic acid, a triterpenoid compound, inhibits the M2 polarization of human monocyte-derived macrophages (HMDM). In the present study, we examined whether oleanolic acid (OA), a triterpenoid compound whose structure is similar to corosolic acid, also shows inhibitory effects on M2 polarization in HMDM. OA significantly inhibited the expression of CD163, one of the phenotype markers of M2 macrophages, as well as suppressed the secretion of IL-10, one of the anti-inflammatory cytokines preferentially produced by M2 macrophages, thus suggesting that OA suppresses the M2 polarization of macrophages. Furthermore, OA inhibited the proliferation of U373 human glioblastoma cells, and the activation of signal transducer and activator of transcription-3 (STAT3) in both human macrophages and glioblastoma cells. These results indicate that OA suppresses the M2 polarization of macrophages and tumor cell proliferation by inhibiting STAT3 activation. Therefore, OA may be a potentially new agent that can be used for the prevention and treatment of various malignant tumors, including glioma.

Introduction

Macrophages infiltrating in cancer tissues are referred to as tumor-associated macrophages (TAMs) and they are considered to be closely involved in the development of the tumor microenvironment (1-3). After the functions of alternatively activated (M2) macrophages were demonstrated, many researchers have focused on this macrophage phenotype in the pathogenesis of various disorders (4).

M2 macrophages are known to be associated with anti-inflammatory functions and angiogenesis in the tumor microenvironment (4,5). We previously demonstrated that CD163 is a useful marker for detecting M2 cells on paraffin-embedded surgical specimens (6), and high infiltration of M2 TAMs are associated with a poor clinical prognosis in patients with high grade glioma, cholangiocarcinoma, angioimmunoblastic T cell lymphoma, and renal cell carcinoma (7-9). Similar results have been reported in melanoma, follicular lymphoma, leiomyosarcoma, and pancreatic cancer (10-12). Therefore, it is speculated that the inhibition of macrophage polarization toward the M2 phenotype could represent a new strategy for anticancer therapy.

In our recent study, we prepared 130 purified compounds from natural products, and screened them for inhibitory effects on the M2 polarization of human monocyte-derived macrophages (HMDM). In that screening, we observed that corosolic acid (CA), a triterpenoid compound, significantly inhibited the M2 polarization of macrophages and glioblastoma cell proliferation by suppressing the activation of STAT3 (13), thus suggesting that CA is a potentially useful candidate agent for cancer immunotherapy.

OA is structurally similar to CA, and is contained in several foods and medicinal plants, and possesses various biological properties, including anti-diabetic, anti-bacterial and anti-oxidative activities (14,15). In this study, we examined the effect of oleanolic acid (OA), a major oleanane-type trterpenoid, on macrophage polarization and glioblastoma cell proliferation in order to identify additional potentially useful candidate anti-cancer agents other than CA.

Materials and methods

Cells and cell culture conditions. The human glioblastoma cell lines, U373-MG (U373) and THP-1 macrophages were

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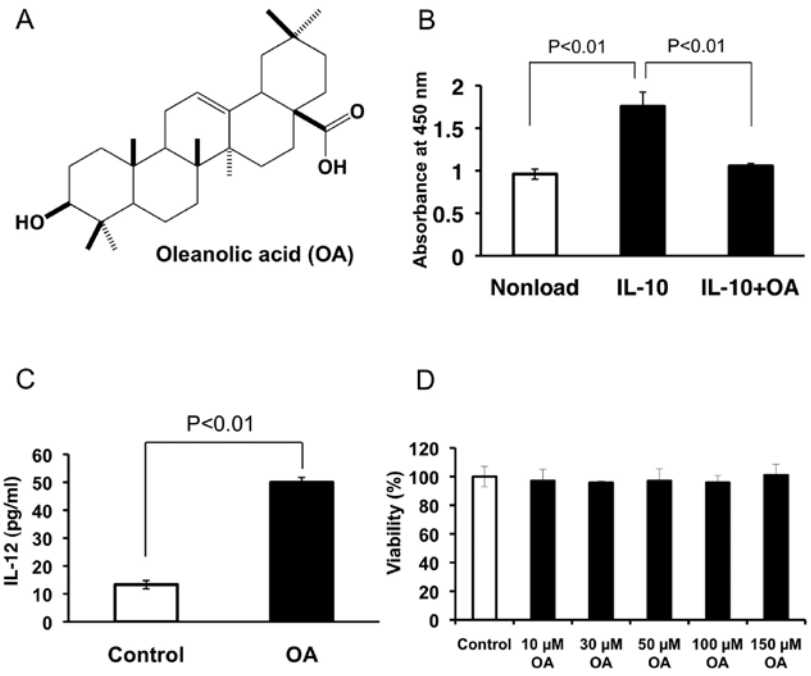

Figure 1. The effects of oleanolic acid on IL-10-induced M2 polarization in HMDM. The chemical structure of oleanolic acid is shown (A). HMDM ($5x10^4$ cells per well of 96-well plates) were incubated with oleanolic acid (30 μM) during the incubation with IL-10 (20 nM) for 24 h, followed by determination of the CD163 expression by Cell-ELISA (B). HMDM ($5x10^4$ cells per well of 96-well plates) were incubated with oleanolic acid (30 μM) for 24 h, followed by determination of the IL-12 secretion by ELISA (C). HMDM were incubated with the indicated concentrations of oleanolic acid for 24 h, followed by the determination of cell proliferation by the WST-8 assay (D).

purchased from American Type Culture Collection (Manassas, VA) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 mg/ml sodium pyruvate. These cells were regularly tested and were found to be negative for Mycoplasma contamination and tumor culture supernatants (TCSs) were prepared as described previously (9).

Peripheral blood mononuclear cells were obtained from healthy volunteer donors. Informed written consent was obtained from all healthy donors. The cells were plated in plates and dishes for 1 h and non-adherent cells were removed by gentle washing with PBS. The remaining monocytes were cultured with GM-CSF (10 ng/ml, Wako, Tokyo, Japan) for 5 days in order to differentiate macrophages (6).

Determination of the inhibitory effect of oleanolic acid on CD163 expression. HMDM ($1x10^4$ cells per well of a 96-well plate) were incubated with or without OA for 24 h after treatment with IL-10 (20 nM) or TCS for 2 days, followed by the determination of CD163 expression by Cell Enzyme-linked Immunosorbent Assay (Cell-ELISA) as described previously (13).

Determination of the inhibitory effect of oleanolic acid on IL-10, and IL-12 secretion. HMDM and THP-1 macrophages ($1x10^4$ cells per well of 96-well plate) were stimulated with LPS (100 ng/ml) for 24 h after incubation with oleanolic acid (30 μM) for 24 h in the presence of TCS, followed by determination of IL-10 and IL-12 secretion by means of an ELISA kit (eBioscience, San Diego, CA).

Immunohistochemistry. Cell block specimens were fixed in 10% neutral buffered formalin and then were embedded in paraffin as described previously (16). Briefly, the sections were deparaffinized in xylene and rehydrated in a graded ethanol series. After the reaction of anti-phosphorylated STAT3 antibody (D3A7), the samples were incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit antibody (Nichirei, Tokyo, Japan). The reaction was visualized by the use of the diaminobenzidine substrate system (Vector, Burlingame, CA).

STAT3 activation assay. STAT3 activation was determined using by measuring the increased expression of the phosphorylated STAT3 by Western blot analysis as described previously (13). Briefly, the solubilized HMDM were run on a 10% SDS-polyacrylamide gel and transferred to a polyvinylidine fluoride (PVDF) transfer membrane (Millipore, Bedford, MA). To detect phosphorylated STAT3, the membranes were exposed to an anti-phosphorylated STAT3 antibody (D3A7) (17) and visualized by horseradish peroxidase-conjugated anti-rabbit IgG antibody with ECL Western blotting detection reagent (CE Healthcare Bio-Sciences). To detect STAT3, the membranes were exposed to an anti-STAT3 antibody (sc-8019; Santa Cruz Biotechnology) (18) and visualized by horseradish peroxidase-conjugated anti-mouse IgG antibody with ECL Western blotting detection reagent. These membranes were re-blotted with an anti-β-actin antibody as an internal calibration control.

Cell proliferation assay. Briefly, $1x10^4$ U373 cells were cultured in a 96-well plate in quadruplicate before treatment. The cells

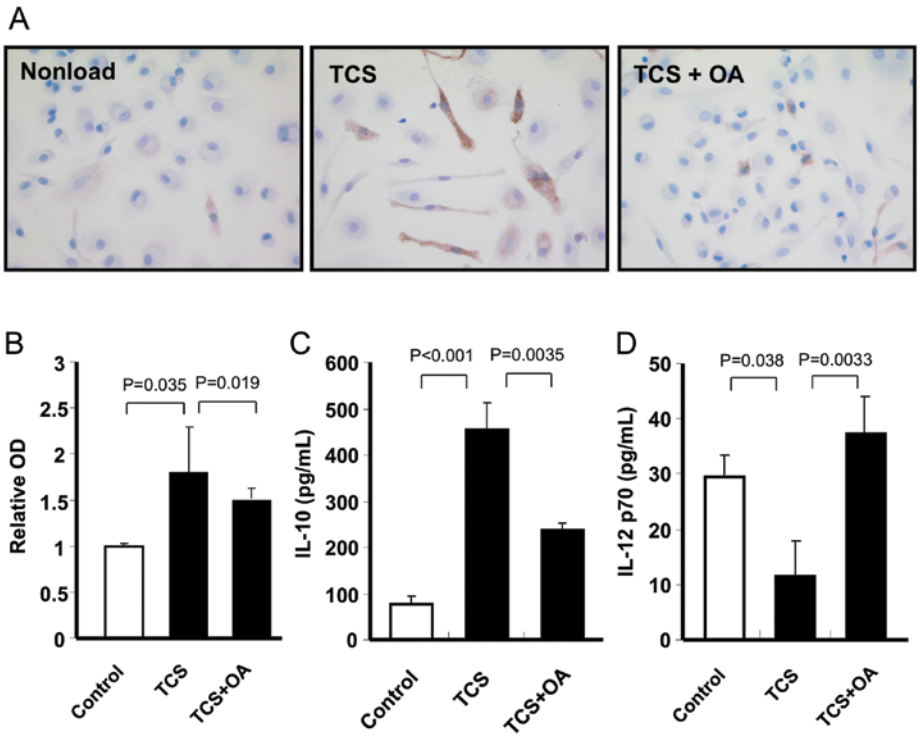

Figure 2. The effects of oleanolic acid on TCS-induced M2 polarization in HMDM. HMDM ($5x10^4$ cells per well of 96-well plates) were incubated with oleanolic acid (30 μM) during the incubation with TCS for 24 h, followed by determination of CD163 expression by an immunohistochemical analysis (A) and Cell-ELISA (B). The HMDM were then stimulated with LPS (100 ng/ml) for 24 h after the incubation with oleanolic acid (30 μM) for 24 h in the presence of TCS, followed by the determination of IL-10 (C) and IL-12 (D) secretion by ELISA.

were then cultured in the presence of oleanolic acid. Cell viability was determined by using the WST assay (WST-8 cell counting kit; Dojin Chemical, Kumamoto, Japan) according to the manufacturer's protocol.

Statistics. All data are representative two or three independent experiments. Data are expressed as the means ± SD. Differences between the groups were examined to determine statistical significance using the Mann-Whitney U test and the non-repeated measures ANOVA. A p-value <0.05 denoted the presence of a statistically significant difference.

Results

The effects of OA on M2 macrophage polarization. We first measured the effect of OA (Fig. 1A) on IL-10-induced CD163 expression as an M2 phenotype marker in HMDM. We observed that OA significantly suppressed the IL-10-induced CD163 expression (Fig. 1B), and also induced the secretion of IL-12, a M1 phenotype marker, in HMDM (Fig. 1C). However, OA caused no morphological changes or cytotoxic effects in the HMDM, even at 150 μM (Fig. 1D). Next, we measured the effects of OA on the expression of CD163 and secretion of IL-10 and IL-12 by HMDM induced by the tumor culture supernatant (TCS) of the U373 glioblastoma cell line. Stimulation with TCS increased the CD163 expression (Fig. 2A and B) and IL-10 secretion (Fig. 2C), and decreased IL-12 secretion (Fig. 2D), in the HMDM. Under the employed assay conditions, OA significantly suppressed the TCS-induced CD163 expression (Fig. 2A and B) and IL-10 secretion (Fig. 2C), and enhanced the IL-12 secretion that was reduced by TCS treatment (Fig. 2D). These data strongly indicate that OA inhibits the M2 polarization of HMDM.

The effects of OA on the JAK-STAT signaling pathway in human macrophages. Since activation of STAT3 contributes to the M2 polarization of macrophages (19,20), we next investigated the effect of OA on IL-10- and TCS-induced STAT3 activation in human macrophages. As shown in Fig. 3A, IL-10 induced STAT3 activation in HMDM. Under the employed assay conditions, OA significantly inhibited the IL-10-induced STAT3 activation (Fig. 3A). Furthermore, OA also inhibited TCS-induced JAK and STAT3 activation in THP-1 macrophages (Fig. 3B and C). These results suggest that OA inhibits the M2 polarization of human macrophages by suppressing the JAK-STAT signaling pathway.

The effects of OA on STAT3 activation and tumor proliferation in glioblastoma cells. It is clear that activation of STAT3 is critically involved in tumorigenesis (21,22), and STAT3 is considered to be an important target molecule for anticancer therapy, including for glioblastoma (23,24). Therefore, we also investigated the effects of OA on STAT3 activation in glioblastoma cells. As shown in Fig. 4A, STAT3 was constantly activated in U373 glioblastoma cells. Under the assay conditions, OA significantly inhibited STAT3 activation (Fig. 4A). Furthermore, OA significantly suppressed glioblastoma cell proliferation at concentrations of 30 μM and higher (Fig. 4B). These data suggest that OA suppresses glioblastoma cell proliferation by inhibiting STAT3 activation.

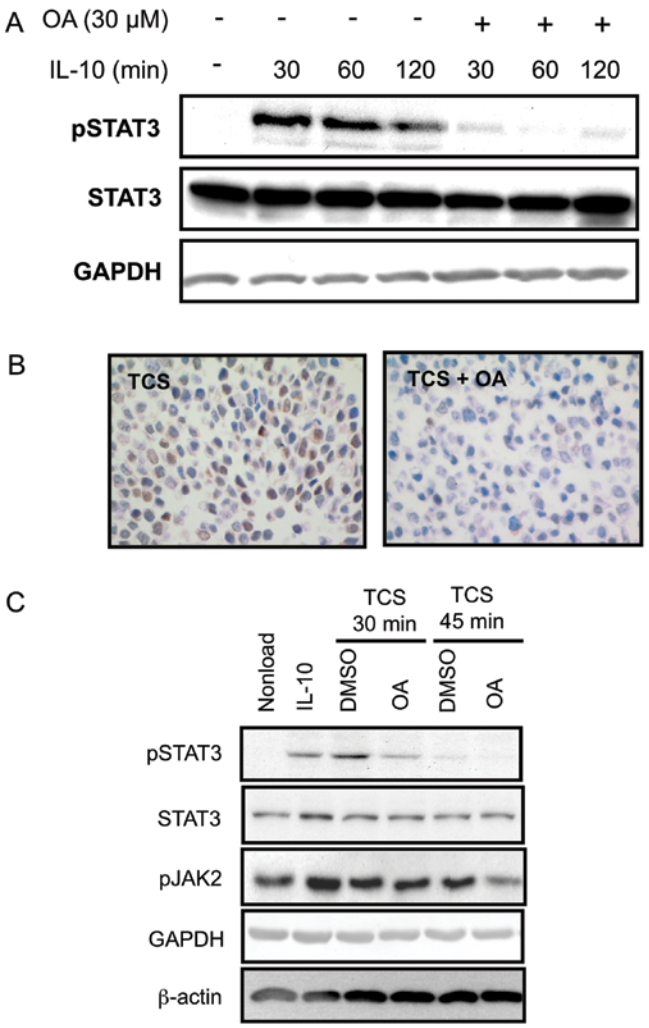

Figure 3. The effects of oleanolic acid on STAT3 activation in human macrophages. HMDM were incubated with oleanolic acid (30 μM) during an incubation with IL-10 (20 nM) for 30, 60 and 120 min, followed by determination of the expression of phosphorylated STAT3, STAT3 and GAPDH by a Western blot analysis (A). THP-1 macrophages were incubated with oleanolic acid (30 μM) during an incubation with TCS for 3 h, followed by determination of the phosphorylated STAT3 expression by immunohistochemical analysis (B). THP-1 macrophages were incubated with oleanolic acid (30 μM) during an incubation with TCS for 30 and 45 min, followed by determination of the expression of phosphorylated STAT3, STAT3, phosphorylated JAK, β-actin and GAPDH by a Western blot analysis (C).

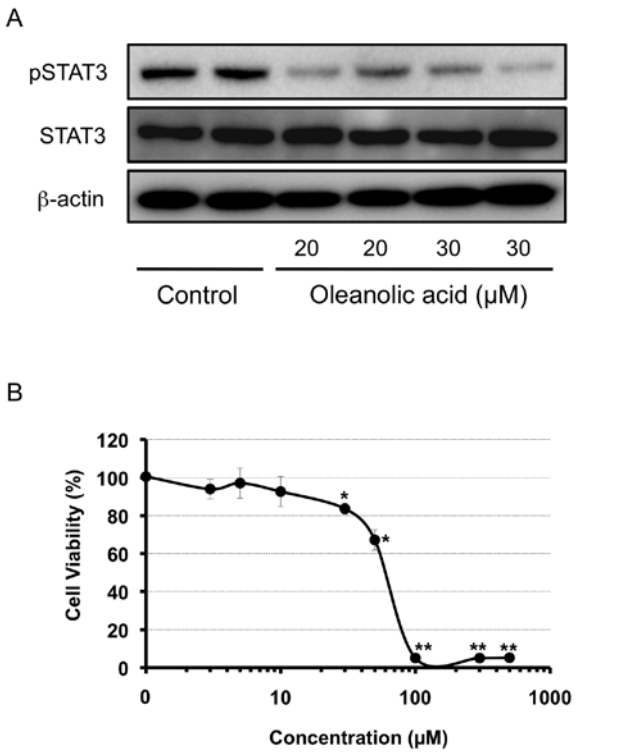

Figure 4. The effects of oleanolic acid on cell proliferation and STAT3 activation in glioblastoma cells. U373 cells were incubated with the indicated concentrations of oleanolic acid for 3 h, followed by determination of the expression of phosphorylated STAT3, STAT3 and β-actin by a Western blot analysis (A). U373 cells were incubated with the indicated concentrations of oleanolic acid for 24 h, followed by the determination of cell proliferation by the WST-8 assay (B). The data are presented as the means ± SD. *P<0.01, **P<0.001 vs. control.

Discussion

M2 TAMs release many proangiogenic cytokines and growth factors, such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), colony stimulation factor-1 (CSF-1), platelet-derived growth factor (PDGF), and basic fibroblast growth factor, which promote tumor progression. They also produce arginase-1, IL-10, and transforming growth factor-β (TGF-β), which inhibit the antitumor function of T cells and natural killer cells (1-3). These cytokines are well known to be induced by STAT3 activation (14). In a murine model of glioma, STAT3 inhibition induced the production of pro-inflammatory cytokines from TAMs, and resulted in tumor growth inhibition (25,26). In human glioma, a STAT3 inhibitor recovered the expression of costimulatory molecules and pro-inflammatory cytokines on peripheral macrophages and TAMs and resulted in the enhancement of immune responses (27). These findings indicate the significance of STAT3 activation in the cell-cell interactions between glioma cells and TAMs.

STAT3 is involved not only in macrophage differentiation, but also in tumor cell proliferation (28). In glioma, STAT3 activation is necessary for the proliferation of glioma cells and glioma stem cells (29). STAT3 activation in glioma cells is closely related to a poor clinical prognosis in patients with grade III glioma (30). Therefore, STAT3 is considered to be a target molecule in patients with glioma (31). In addition, STAT3 activation in tumor cells is considered to cause tumor cell resistance to anti-cancer therapies, such as chemotherapy and radiotherapy (32). We also previously demonstrated that the STAT3 inhibitor CA enhanced the efficacy of chemotherapeutic agents against glioblastoma cells.

In this study, we showed that OA significantly suppressed the JAK-STAT3 activation in human macrophages and glioblastoma cells, and inhibited the macrophage polarization into the M2 phenotype, and also decreased the proliferation of glioblastoma cells. Inhibition of macrophage differentiation into the M2 phenotype is suggested to increase the immune response in patients with glioblastoma. OA might also directly suppress the proliferation of glioblastoma cells, and increase their sensitivity to chemotherapy or radiotherapy. These data suggest that OA may be a potentially useful new compound for anticancer therapy.

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