VEGF induces phosphorylation of STAT3 through binding VEGFR2 in ovarian carcinoma cells in vitro

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Summary

VEGF plays a key role in ovarian carcinoma. Recent studies have shown that expressions of VEGF and its receptors were correlated with signal tranducer phosphorylation and activators of transcription 3(p-STAT3) in ovarian carcinoma. The aim of this study was to investigate the effects of STAT3 phosphorylation on VEGF signaling pathways in ovarian carcinoma cells. We selected an ovarian carcinoma cell line Caov-3 as a target cell that co-expressed VEGFR2 and p-STAT3. We detected expressions of p-STAT3 in Caov-3 induced by VEGF with different concentrations and for different effect times by immunocytochemistry and Western Blot. A concentration of 50 ng/ml VEGF was enough to increase phosphorylation of STAT3, and at 30 min, the p-STAT3 level reached the peak and showed nuclear translocation of p-STAT3 from the cytoplasm to the nucleus. These effects could be overcome by a small peptide (ATWLPPR) specific for VEGFR2. Taken together, VEGF-induced phosphorylation and nuclear translocation of STAT3 and ATWLPPR could effectively block the VEGF effects, suggesting that phosphorylation of STAT3 participates in VEGF signal transduction via VEGFR2 in ovarian carcinoma cells.

Key words: Ovarian neoplasm; VEGF; VEGFR; STAT; Signaling.

Introduction

Ovarian carcinoma is a disease with the poorest prognosis in gynecological malignancies. Widespread and great accumulation of ascites have been found in patients with ovarian carcinoma at the first diagnosis, but the mechanism remains unclear. Excessive high levels of VEGF in ascites may be involved [1]. VEGF is an endothelial cell-specific mitogen. It can be produced by various normal cells and cancer cells [2]. Up to now, many studies have shown that VEGF stimulates proliferation, migration, angiogenensis of the endothelial cells and increases the microvascular permeability [3]. It is believed that VEGF receptors (VEGFRs), which mediate VEGF biological effects and some kinds of signaling pathways, including MAPK [4-6], PI3K [7, 8], PLC-γ and ion pathways, participate in this procedure in endothelial cells. VEGF belongs to the family of growth factors. Recent studies also showed that growth factors such as EGF and PDGF could activate STAT signal transduction [9-11].

STAT proteins were originally identified as mediating interferon (IFN)-α and IFN-γ. As a group of transcription factors, they induced downstream gene transcription. Nonactivated STATs are located in cytoplasm, but activated proteins may translocate to the nucleus where they bind to target genes, such as oncogene fos, jun, and myc, etc., transduce signals to the transcriptional region of DNA, regulate the gene expression [12, 13] and then change the endothelial cell biological characteristics.

Taken together, tumor cells produce VEGF. Through binding to VEGFRs on vascular endothelial cells, VEGF exerts its biological effects through STATs signal transduction. However, recent studies showed that VEGFRs were also expressed on various kinds of tumor cells, e.g. human neuroblastoma, prostate cancer, breast cancer, pancreatic cancer, malignant melanoma and ovarian carcinoma [14-16]. It is thus suggested that there is not only a paracrine loop of VEGF in endothelial cells to promote angiogenesis, but also an autocrine loop in tumor cells to directly induce tumor growth and prevent apoptosis [16, 17]. But how VEGF affects tumor cells themselves and induces intracellular signal transduction, and whether its signal pathway is similar to that in endothelial cells, especially, whether the STAT signal pathway is involved, are not yet clear.

Constitutive activation of STAT3 has been found in a lot of malignancies including breast [9, 18], ovarian [19], prostate cancer [20] and amyloid marrow lymphoma [21], etc. In contrast, a benign ovarian tumor cell line, A2780, and normal ovarian surface epithelial cells had little STAT3 phosphorylation [22, 23]. A further study showed that ovarian carcinoma cells with activated STAT3 overexpressed Bcl-x(L) and cyclin D1, whereas A2780 cells without activated STAT3 only slightly expressed the two genes [22]. Experiments on naked mice also revealed that abnormal activation of STAT3 could result in cell transformation and induce oncogenesis [10, 24-26]. All the above results suggest that activation of STAT3 is a common event during oncogenic transformation and is associated with malignant behavior. Considering STAT3 function, we propose that VEGF signal transduction in malignant cells probably involves STAT3 phosphorylation.

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In the study, the expressions of p-STAT3 (phosphorylated STAT3) induced by VEGF with different concentrations and for different effect-times in ovarian carcinoma cell lines in vitro were detected by immunocytochemistry and Western Blot. Furthermore, inhibition of p-STAT3 induced by VEGF was observed through blocking the binding of VEGF and VEGFR2 with a peptide specific for VEGFR2. The aim of our study was to investigate the effects of phosphorylated STATs on VEGF signaling pathways in ovarian carcinoma cells.

Materials and Methods

Reagents

VEGF was purchased from Sigma biotechnological company; Peptide (ATWLPPR) was synthesized by Shengyou Biotechnology of China, LTC; Polyclonal antibodies against p-STAT3, VEGFR2 and β-actin were purchased from Santa Cruz Biotechnology, Inc; HRP marked anti-goat and anti-rabbit secondary antibodies were purchased from Santa Cruz Biotechnology, Inc.

Cell culture

Epithelial ovarian carcinoma (EOC) cell lines SKOV-3 and Caov-3 were obtained from American Type Culture Collection and 3AO was obtained from the cell bank of Shanghai Institute of Biochemistry and Cell Biology of the Chinese Academy of Science. The cells were cultured in complete medium: RPMI 1640 (Life Technologies, Inc.), supplemented with 15% heat-inactivated FCS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin (Life Technologies, Inc.). Eighty-five or 90% percent confluent cells were cultured in serum-free medium for 16~18 hours and then treated by VEGF with different concentrations and for different effect-times.

Experimental design

The expressions of p-STAT3 in three cell lines treated by VEGF were detected, and the cell lines without VEGF treatment as controls. A cell line with the highest expression of p-STAT3 induced by VEGF was selected for further studies. P-STAT3 expressions of the cells incubated with VEGF with different concentrations (0, 5, 25, 50, 100 ng/ml) for 30 min and with 50 ng/ml for different effect-times (0, 15, 30, 45, 60 min) were detected. The inhibition of p-STAT3 expression induced by VEGF (50 ng/ml) was further observed through blocking VEGF binding VEGFR2 by a peptide (ATWLPPR) with different concentrations (0, 40, 80, 160, 320 μ M) for 30 min and with 80 μM for different effect-times (0, 15, 30, 45, 60 min).

Immunocytochemistry

Incubation of VEGF was terminated by washing the cells twice in ice-cold phosphorylate-buffered saline. The cells were fixed with 2.5% glutaric dialdehyde for 10 min. Intracellular translocation of p-STAT3 in response to VEGF was observed by immunocytochemistry following a procedure described previously. Grade evaluation standard followed one described previously [27].

Extraction of proteins

Pretreatment of the cells with 40 μ M $NaVO_3$ and incubation of VEGF and peptide were terminated by washing the cells twice in ice-cold phosphate-buffered saline. Cell lysates were obtained using lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 1.0 mM DTT, 100 μ g/ml PMSF, 1% NP-40, 200 μ M $NaVO_3$, 1 μ g/ml Aprotinin). The cells were incubated with cell lysates on ice for 30 min, scraped and centrifugated at 12,000 rpm at 4°C. The supernatant was collected and stored at -20°C.

Western Blot

Protein extracts were separated on 12% SDS polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Immunoblotting using polyclonal antibodies against p-STAT3 and β-actin antibodies and signal was detected using peroxidase-conjugated secondary antibody followed by development using enhanced electrochemiluminescence system.

Statistical Analysis

The results were semi-quantitified by grade evaluation for immunocytochemical staining and Quantity One Software (Bio-Rad, Inc.) for Western blot. Oneway ANOVA and univariate analysis of variance were used for statistical analysis. All data were managed with SPSS 10.0 for windows. For all statistical analyses, the level with a probability of 0.05 was considered significant. Data were presented as the mean ± SE.

Results

Expression of p-STAT3 and VEGFR2 in EOC cell lines

VEGFR2 and p-STAT3 were found to be co-expressed in all three cell lines. The p-STAT3 was located in the cytoplasm and nucleus, and VEGFR2 was mainly located on the surface of cell membranes and partially in cytoplasm. Caov-3 was selected as a model for further study because it co-expressed both VEGFR2 and p-STAT3 and expressed the highest p-STAT3 after VEGF incubation (50 ng/ml, 30 min).

Expression of p-STAT3 in Caov-3 induced by VEGF

Localization

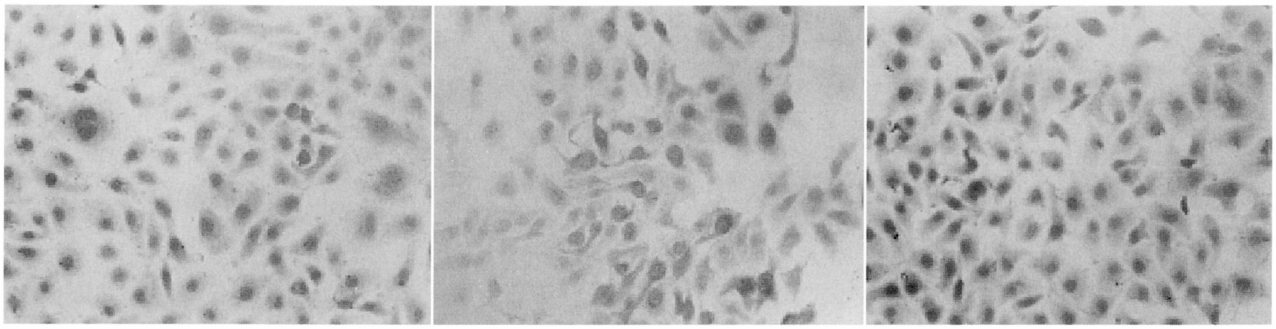
Expression of p-STAT3 in Caov-3 induced by VEGF with different stimulation concentrations was increased including both intensity of staining and the number of positive-stained cells. The translocation of p-STAT3 was found depending on the time of VEGF incubation (Figure 1).

Semi-quantitative analysis for different VEGF concentrations

P-STAT3 expression of Caov-3 was significantly elevated following increased VEGF concentration ($p = 0.000$) (Figure 2).

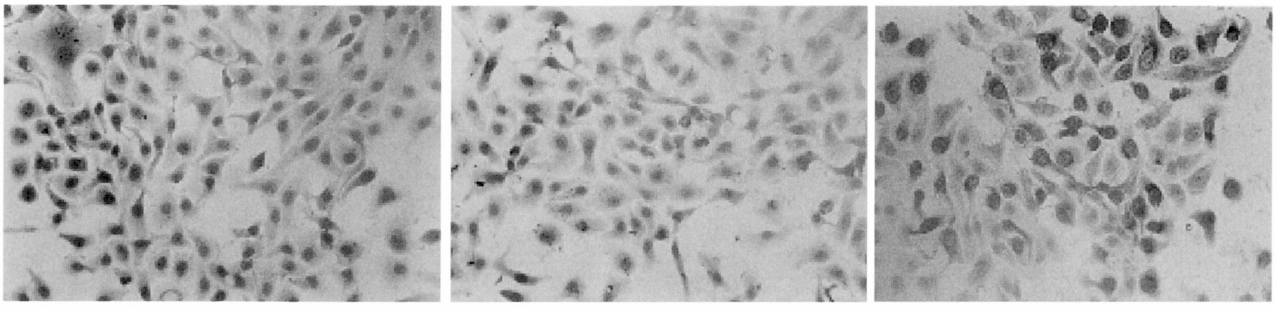
Semi-quantitative analysis for different VEGF effect-times

VEGF induced different levels of p-STAT3 expression in Caov-3 for different effect-times, and there were significant differences among different groups (immunocytochemistry: $p = 0.000$, Western blot: $p = 0.001$) (Figure 3).

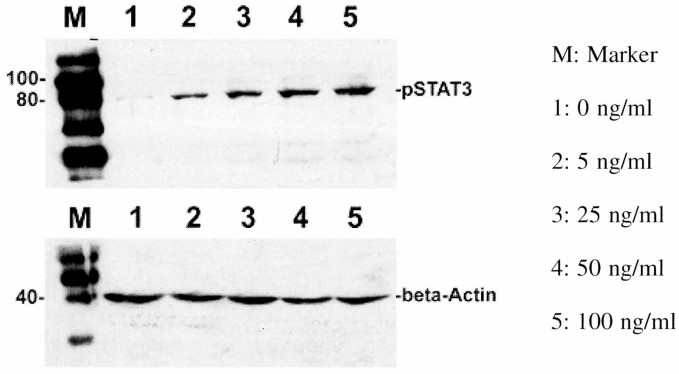


A: 15 min (200 x 1) B: 30 min (200 x 1) C: 60 min (200 x 1)

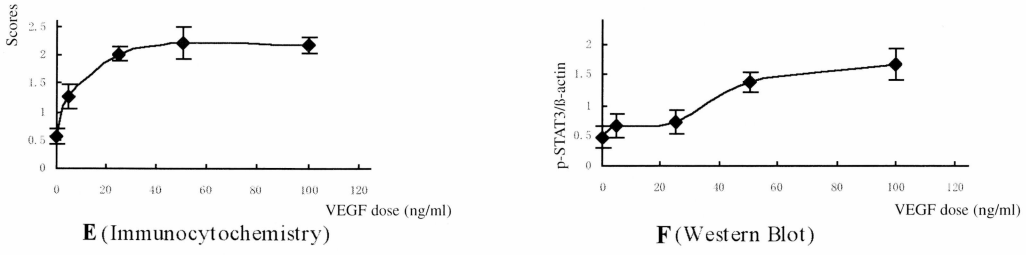
Figure 1. — Translocation of p-STAT3 in Caov-3 induced by VEGF with different effect-times. A: p-STAT3 was located in the cytoplasm and perinuclear region at 15 min; B: in the nucleus at 30 min; C: mainly in cytoplasma again at 60 min.



A: 0 ng/ml (200 x) B: 5 ng/ml (200 x) C: 50 ng/ml (200 x)



D (Western blot)



E (Immunocytochemistry) **F** (Western Blot)

Figure 2. — Expression of p-STAT3 in Caov-3 induced by VEGF with different concentrations. Expression of p-STAT3 was increased with ascending VEGF concentrations, 50 ng/ml VEGF induced p-STAT3 to reach the maximum. A, B, C: expression of p-STAT3 by immunocytochemistry; D: expression of p-STAT3 by the Western blot method; E, F: dose-dependent curves. Comparison between the two groups showed that there were significant differences of p-STAT3 expression at 0 and 5, 5 and 25 ng/ml of VEGF concentration by immunocytochemistry ($p = 0.000$ and 0.000) and at 25 and 50 ng/ml by Western blot ($p = 0.000$). However no significant differences of p-STAT3 expression existed at 25 and 50 ng/ml by immunocytochemistry ($p = 0.140$) and at 50 and 100 ng/ml by both detection methods (immunocytochemistry: $p = 0.87$, Western blot: $p = 0.052$).

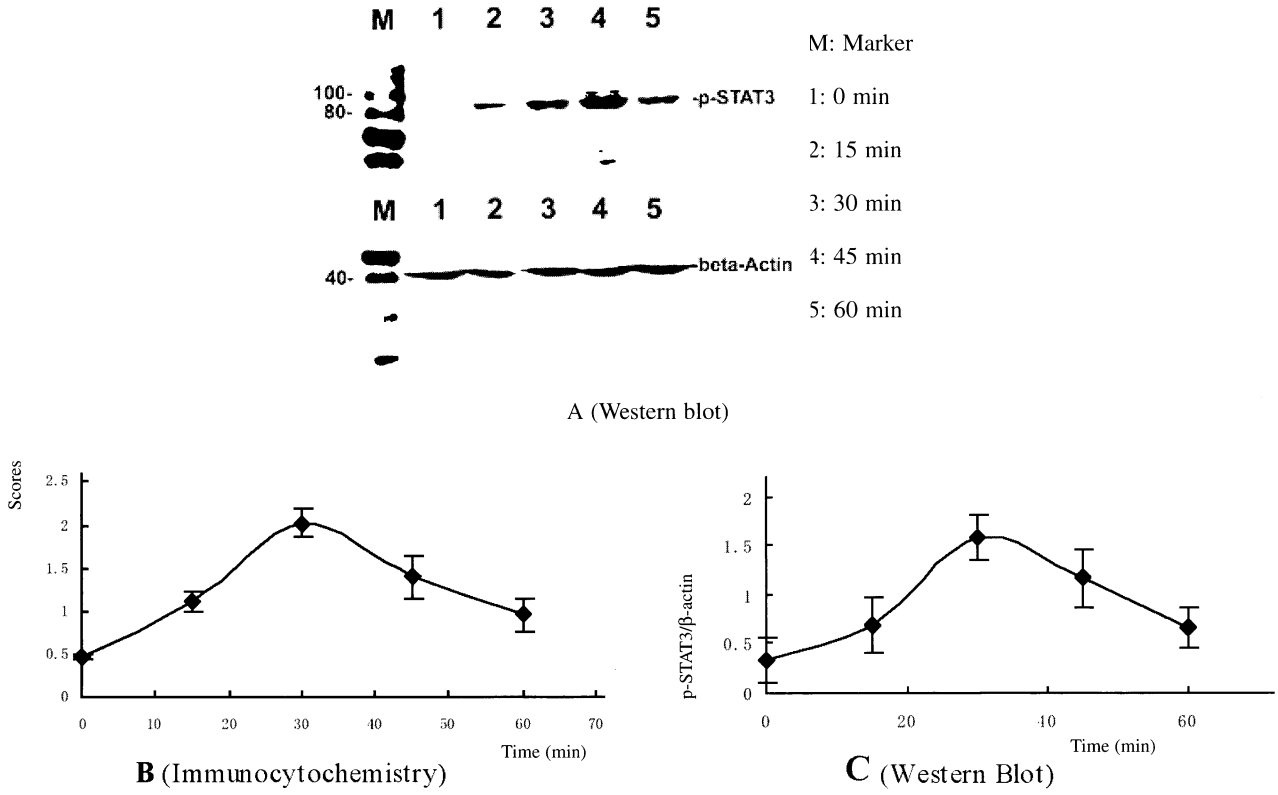


Figure 3. — Expression of p-STAT3 induced by VEGF with different effect-times. A: expression of p-STAT3 in Caov-3 by Western blot; B, C: time-dependent curves. Expression of p-STAT3 reached the peak at 30 min and was significantly higher compared with that at 0 min and 60 min by both detection methods (immunocytochemistry: p = 0.000 and 0.000, Western blot: p = 0.000 and 0.001), but not significantly compared with that at 15 and 45 min; although at 15 and 45 min they were higher than at 0 and 60 min, but not significantly.

Peptide-blocked increase of p-STAT3 induced by VEGF in Caov-3

Peptide with different concentrations blocking VEGF effects

The expressions of p-STAT3 in Caov-3 induced by VEGF were decreased following increased peptide concentration and there were significant differences of p-STAT3 expression among the groups (Immunocytochemistry: p = 0.000, Western blot: p = 0.000) (Figure 4).

Peptide blocking VEGF effects for different effect-times

Expressions of p-STAT3 induced by VEGF were significantly blocked by peptide for different effect-times by both detecting methods (p = 0.000) (Figure 5).

Discussion

STAT3 phosphorylation participates in VEGF signal transduction in ovarian carcinoma cells

Oncogenesis is always associated with abnormal regulation of cell growth procedures. STATs, activator transcription factors obtaining double recognizations, play key roles in regulation of downstream gene expression [28]. VEGF exerts angiogenesis through binding to VEGFRs and inducing phosphorylation of STAT3 in endothelial cells. A concentration of 10 ng/ml was enough to induce significant phosphorylation of STAT3 resulting in regulation of downstream gene expression, thus promoting proliferation and preventing apoptosis [13, 29-31]. However, phosphorylation of STATs in VEGF signaling in tumor cells has not been conclusive. Schaefer LK *et al.* [32] found that co-expression of STAT3 α and VEGFR2 in brain tumors resulted in ligand-independent activation of STAT3 α and was related to increased transcription activity, but they did not explore the relationship between VEGF and STAT3. In our previous study, we detected VEGF, VEGFR and p-STAT3 expression in patients with epithelial ovarian carcinoma by immunocytochemistry and found that there were high co-expressions of VEGF, VEGFR and p-STATs in carcinoma cells. Furthermore the expressions of phosphorylated STAT3 and STAT5 were positively correlated with VEGF and VEGFRs.

In the study, co-expression of p-STAT3 and VEGFR2 in ovarian carcinoma cell lines was detected. Expressions of p-STAT3 in Caov-3 were increased after VEGF stim-

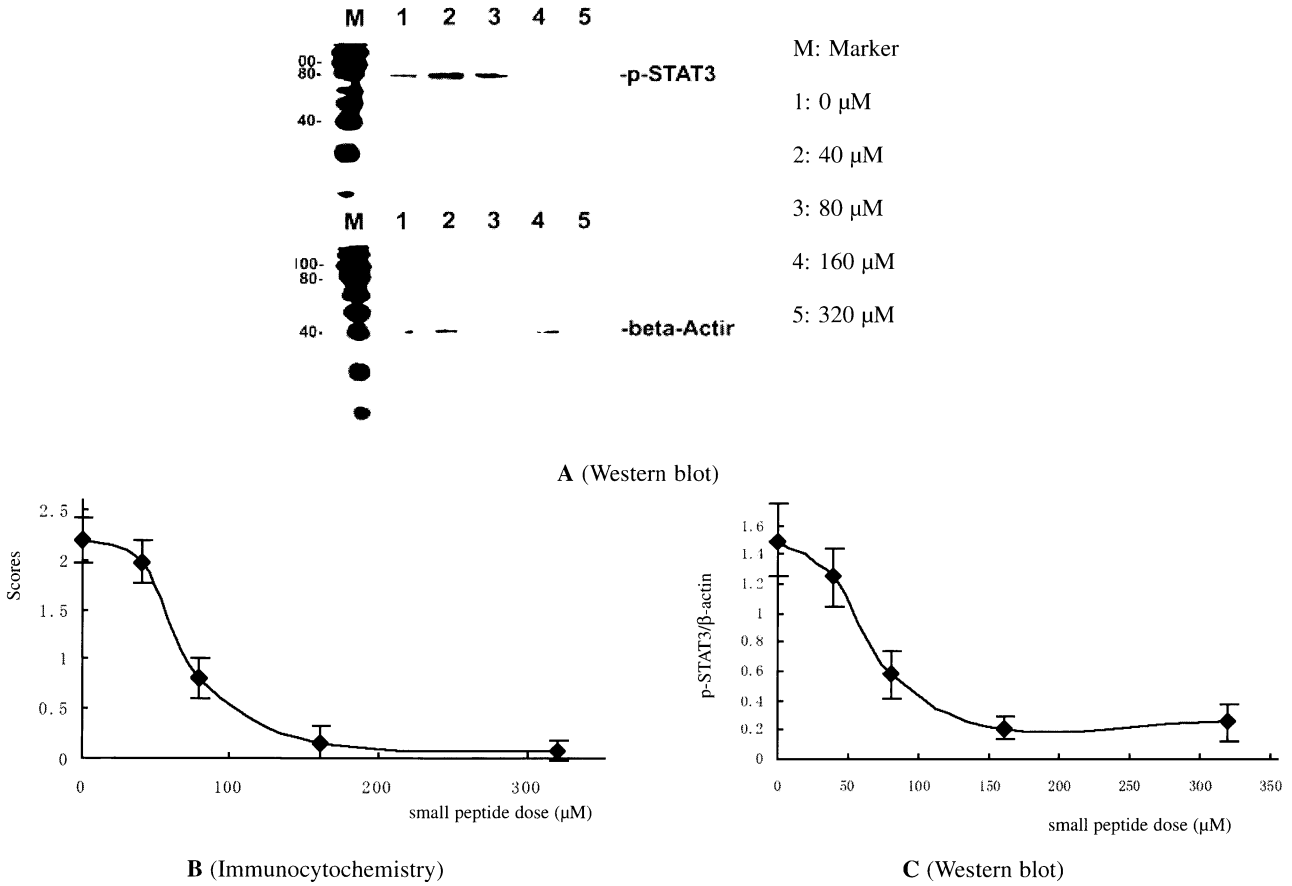
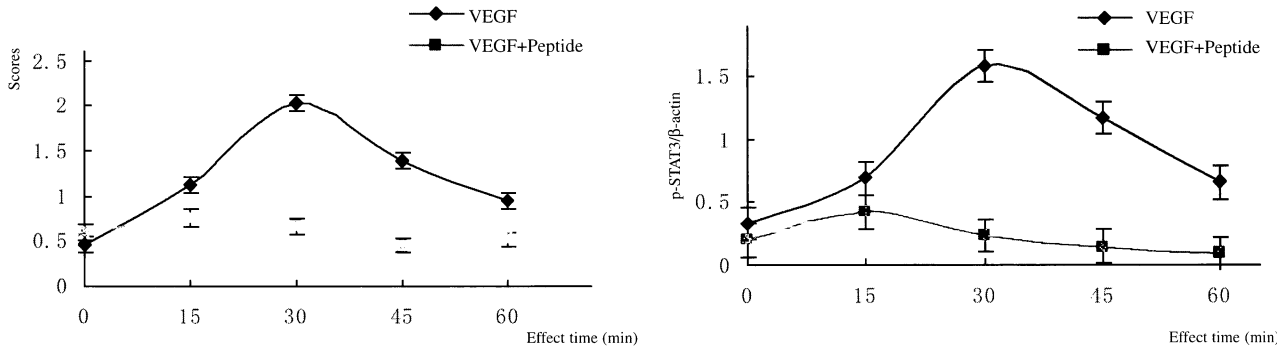


Figure 4. — Expression of p-STAT3 induced by VEGF was blocked by peptides with different concentrations. A: expression of p-STAT3 blocked by peptide using Western blot; B, C: dose-dependent curves. With 80 μM, 160 μM and 320 μM peptide, significant decreases of p-STAT3 expression induced by VEGF (50 ng/ml) were observed compared with the control by both detection methods (immunocytochemistry: p = 0.000, Western blot: p = 0.000), whereas with 160 μM and 320 μM they were more significantly lower than with 80 μM (160 μM vs 80 μM: immunocytochemistry: p = 0.000, Western blot: p = 0.025; 320 μM vs 80 μM: immunocytochemistry: p = 0.000, Western blot: p = 0.040). However there were no differences between 160 μM and 320 μM (immunocytochemistry: p = 0.574, Western blot: p = 0.790).

ulation with different concentrations, indicating that STAT3 phosphorylation participates in VEGF signaling in ovarian carcinoma cell lines. Further observation found that 5 ng/ml of VEGF was enough to induce a significant increase of p-STAT3 expression by immunocytochemistry and 50 ng/ml by both immunocytochemistry and Western blot. Thus, we believe that 50 ng/ml is an efficient concentration to induce phosphorylation of STAT3 in ovarian carcinoma cells in vitro. However, this concentration seems obviously higher than that in endothelial cells. The mechanism of the discordance between the two cell types is unclear. The different numbers of receptors on the two type cells might be involved. Furthermore, we could not find a significant difference of p-STAT3 expression between with 50 ng/ml and 100 ng/ml concentrations of VEGF. It is indicated that too high a concentration of VEGF stimulation does not promote further activation of STAT3 in ovarian carcinoma cells in vitro.

Not only is phosphorylation of STAT3 induced by VEGF related to its concentration, but its also related to the effect-time. In previous studies on endothelial cells, C5b9 and IFN-γ could activate STAT3, with the peak of p-STAT3 expression occurring at 15~30 min and returning to the basic level at 120 min after C5b9 and IFN-γ stimulation [33, 34]. However, another study showed that STAT3 activation could reach a peak at 5 min after stimulation [35]. We found that after VEGF stimulation, p-STAT3 expression started to increase at 15 min, reached the climax at 30 min and then decreased. It indicated that a too long effect-time of VEGF stimulation does not promote any further activation of STAT3. Taken together, our findings suggest that VEGF induces phosphorylation of STAT3 in ovarian carcinoma cells in vitro, but the intensity of its effect is associated with its incubating concentration and effect-time. The mechanisms are not clear and may be due to a negative feedback between VEGF and VEGFRs or the saturation of VEGFRs binding to VEGF, but further investigation is needed.



A (Immunocytochemistry) **B** (Western blot)

Figure 5. — Expression of p-STAT3 induced by VEGF was blocked by peptides for different effect-times. VEGF (50 ng/ml) induced an increase of p-STAT3 expression with a peak at 30 min. However, the peptide (80 μ M) inhibited the expression of p-STAT3 induced by VEGF. The effects occurred at 15 min, obviously at 30 min and maintained until 60 min by both detection methods, but there were no significant differences among the effect-times by either detection method (immunocytochemistry: $p = 0.166$; Western blot: $p = 0.315$).

VEGF induces phosphorylated STAT3 translocating into nuclei

STAT is a member of the cytoplamic protein family, but is able to form a dimmer and translocate into a nucleus after activated in order to regulate the expression of downstream genes. However, the opinion that activated STAT3 translocated into nuclei has been debated in previous studies. Bartoli *et al.* found that VEGF activated STAT1 and STAT6, and induced them to translocate into nuclei in bovine aortic endothelial cells, but not translocation of activated STAT3. Similarly in keratocytes, IL-4 induced STAT3 phosphorylation, but phosphorylated STAT3 was not found to translocate into nuclei [35]. However, most reports have concurred that activated STAT3 could translocate into nuclei. For example, C5b9 induced phosphorylated STAT3 can translocate into nuclei in endothelial cells 15 min after stimulation [33]. Similar results have been observed in various tumor cells such as breast cancer, fibroblastoma, lymphoma, etc. [9, 36, 37]. However no study on translocation of activated STAT3 induced by VEGF in tumor cells has been reported.

In the study, we first found that VEGF induced STAT3 phosphorylation and phosphorylated STAT3 translocated into nuclei. It is morphologically suggested that activated STAT3 induced by VEGF participates in VEGF signaling transduction and regulates the transcription of downstream genes. Moreover, we also observed that the staining idensity of activated STAT3 was significantly decreased at 60 min, and the most positive granules were again located in the cytoplasm. This morphologic evidence further supports our opinion that VEGF-induced STAT3 activation in vitro is dependent on its effect-times.

VEGF induces phosphorylatation of STAT3 mediated by VEGFR2

Although the affinity of VEGFR1 is about 10-fold higher for VEGF than VEGFR2, the latter is considered to be a major receptor that conveys VEGF-induced signaling in endothelial cells. Bartoli *et al.* and Korplelanen *et al.* [13, 38] confirmed that VEGF inducing activation of STATs was mainly mediated by VEGFR2. Our previous studies revealed that the expression of VEGFR2 had a significant correlation with activation of STAT3 in ovarian epithelial carcinoma cells by immunocytochemistry. It was suggested that VEGF-induced STAT activation was probably mediated by VEGFR2 in ovarian carcinoma, but there is no exact evidence.

The peptide (ATWLPPR) is an inhibitor to block VEGF binding to VEGFR2. Binetruy *et al.* [39] identified the peptide able to block VEGF-KDR(VEGFR2) interaction by a phage epitope library, screening affinity by membrane-expressed KDR or by an anti-VEGF neutralizing monoclonal antibody. ATWLPPR can completely abolish VEGF binding to cell-displayed KDR. It was reported that this effect led to the inhibition of the VEGF-mediated proliferation of human vascular endothelial cells in vitro and abolished totally VEGF-induced angiogenesis in a rabbit corneal model [39]. In the study, we observed that ATWLPPR effectively inhibited the increase of p-STAT3 expression induced by VEGF in Caov-3. A further analysis revealed that the effects of inhibition were related to peptide concentration and effect-time. This phenotype of inhibition supports the theory that VEGF-induced p-STAT3 activation is mediated by VEGFR2. Our results also indicate that the small peptide may have clinical application potential as an antagonist of VEGFR2.

References

[1] Santin A.D., Hermonat P.L., Ravaggi A., Cannon M.J., Pecorelli S., Parham G.P.: "Secretion of vascular endothelial growth factor in ovarian cancer". *Eur. J. Gynaecol. Oncol.*, 1999, *20*, 177.

[2] Chen C.A., Cheng W.F., Lee C.N., Chen T.M., Kung C.C., Hsieh F.J. *et al.*: "Serum vascular endothelial growth factor in epithelial ovarian neoplasms: correlation with patient survival". *Gynecol. Oncol.,* 1999, *74,* 235
[3] Carmeliet P., Jain R.K.: "Angiogenesis in cancer and other disease". *Nature*, 2000, *407*, 249
[4] Takahashi T., Ueno H., Shibuya M.: "VEGF activates protein kinase C-dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells". *Oncogene,* 1999, *18,* 2221
[5] Ozaki H., Seo M.S., Ozaki K., Yamada H., Yamada E., Okamoto N. *et al.*: "Blockade of vascular endothelial cell growth factor receptor signaling is sufficient to completely prevent retinal neovascularization". *Am. J. Pathol.,* 2000, *56,* 697
[6] Wu L.W., Mayo L.D., Dunbar J.D., Kessler K.M., Baerwald M.R., Jaffe E.A. *et al.*: "Utilization of distinct signaling pathways by receptors for vascular endothelial cell growth factor and other mitogens in the induction of endothelial cell proliferation". *J. Biol. Chem.*, 2000, *275,* 5096
[7] Jiang B.H., Zheng J.Z., Aoki M., Vogt P.K.: "Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells". *Proc. Natl. Acad. Sci USA,* 2000, *97*, 1749
[8] Suzuma K., Naruse K., Suzuma I., Takahara N., Ueki K., Aiello L.P. *et al.*: "Vascular endothelial growth factor induces expression of connective tissue growth factor via KDR, Flt1, and phosphatidylinositol 3-kinase-akt-dependent pathways in retinal vascular cells". *J. Biol. Chem.*, 2000, *275*, 40725
[9] Berclaz G., Altermatt H.J., Rohrbach V., Siragusa A., Dreher E., Smith P.D.: "EGFR dependent expression of STAT3 (but not STAT1) in breast cancer". *Int. J. Oncol.*, 2001, *19,* 1155
[10] Song J.I., Grandis J.R.: "STAT signaling in head and neck cancer". *Oncogene,* 2000, *19,* 2489.
[11] Wang Y.Z., Wharton W., Garcia R., Kraker A., Jove R., Pledger W.J.: "Activation of Stat3 preassembled with platelet-derived growth factor beta receptors requires Src kinase activity". *Oncogene,* 2000, *19*, 2075.
[12] Enaida H., Kabuyama Y., Oshima Y., Sakamoto T., Kato K., Kochi H. *et al.*: "VEGF-dependent signaling in retinal microvascular endothelial cells". *J. Med. Sci,* 1999, *45,* 77.
[13] Bartoli M., Gu X., Tsai N.T., Venema R.C., Brooks S.E., Marrero M.B. *et al.*: "Vascular endothelial growth factor activates STAT proteins in aortic endothelial cells". *J. Biol. Chem.,* 2000, *275,* 33189.
[14] Meister B., Grunebach F., Bautz F., Brugger W., Fink F.M., Kanz L. *et al.*: "Expression of vascular endothelial growth factor and its receptors in human neuroblastoma". *Eur. J. Cancer,* 1999, *35*, 445.
[15] Ferrer F.A., Miller L.J., Lindquist R., Kowalczyk P., Laudone V.P., Albertsen P.C. *et al.*: "Expression of vascular endothelial growth factor receptor in human prostate cancer". *Urology,* 1999, *54*, 567.
[16] Jackson M.W., Roberts J.S., Heckford S.E., Ricciardelli C., Stahl J., Choong C. *et al.*: "A potential autocrine role for vascular endothelial growth factor in prostate cancer". *Cancer Res.,* 2002, *62,* 854.
[17] Masood R., Cai J., Zheng T., Smith D.L., Hinton D.R., Gill P.S.: "Vascular endothelial growth factor (VEGF) is an autocrine growth factor for VEGF receptor-positive human tumors". *Blood,* 2001, *98,* 904.
[18] Lin J., Jin X., Rothman K., Lin H.J., Tang H., Burke W.: "Modulation of signal transducer and activator of transcription 3 activities by p53 tumor suppressor in breast cancer cells". *Cancer Res.,* 2002, *62,* 376.
[19] Burke W.M., Jin X., Lin H.J., Huang M., Liu R., Reynolds R.K. *et al.*: "Inhibition of constitutively active Stat3 suppresses growth of human ovarian and breast cancer cells". *Oncogene,* 2001, *20,* 7925.
[20] Gao B., Shen X., Kunos G., Meng Q., Goldberg I.D., Rosen E.M., *et al.*: "Constitutive activation of JAK-STAT3 signaling by BRCA1 in human prostate cancer cells". *FEBS Lett.*, 2001, *488,* 179.
[21] Benekli M., Xia Z., Donohue K.A., Ford L.A., Pixley L.A., Baer M.R. *et al.*: "Constitutive activity of signal transducer and activator of transcription 3 protein in acute myeloid leukemia blasts is associated with short disease-free survival". *Blood.*, 2002, *99*, 252.
[22] Huang M., Page C., Reynolds R.K., Lin J.: "Constitutive activation of Stat3 oncogene product in human ovarian carcinoma cells" *Gynecol. Oncol.*, 2000, *79,* 67.
[23] Li L., Shaw P.E.: "Autocrine-mediated activation of STAT3 correlates with cell proliferation in bresast carcinoma lines". *J. Biol. Chem.,* 2002, *277,* 17397
[24] Bowman T., Garcia R., Turkson J., Jove R.: "STATs in oncogenesis". *Oncogene*, 2000, *19,* 2474.
[25] Imada K., Leonard W.J.: "The Jak-STAT pathway". *Mol. Immunol.,* 2000, *37,* 1.
[26] Grandis J.R., Drenning S.D., Zeng Q., Watkins S.C., Melhem M.F., Endo S. *et al.*: "Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinogenesis in vivo". *Proc. Natl. Acad. Sci USA,* 2000, *97*, 4227.
[27] Ma Z.Z., Han X., Liu S.F.: "Diagnostic Cytopathology". Henan Scientific and Technical Publishing House, 1st edition, 2000, 823.
[28] Schuringa J.J., Timmer H., Luttickhuizen D., Vellenga E., Kruijer W.: "c-Jun and c-Fos cooperate with STAT3 in IL-6-induced transactivation of the IL-6 response element IRE". *Cytokine,* 2001, *14*, 78.
[29] Shen Y., Devgan G., Darnell J.E., Bromberg J.F.: "Constitutively activated Stat3 protects fibroblasts from serum withdrawal and UV-induced apoptosis and antagonizes the proapoptotic effects of activated Stat1". *Proc. Natl. Acad. Sc. USA*, 2001, *98,* 1543.
[30] Rebbaa A., Chou P.M., Mirkin B.L.: "Factors secreted by human neuroblastoma mediated doxorubicin resistance by activating STAT3 and inhibiting apoptosis". *Mol. Med.,* 2001, *7,* 393.
[31] Epling Burnette P.K., Liu J.H., Catlett-Falcone R., Turkson J., Oshiro M., Kothapalli R. *et al.*: "Inhibition of STAT3 signaling leads to apoptosis of leukemic large granular lymphocytes and decreased Mcl-1 expression". *J. Clin. Invest.,* 2001, *107,* 351.
[32] Schaefer L.K., Ren Z., Fuller G.N., Schaefer T.S.: "Constitutive activation of STAT3α in brain tumors: localization to tumor endothelial cells and activation by the endothelial tyrosine kinase receptor (VEGFR-2)". *Oncogene*, 2002, *21,* 2058.
[33] Niculescu F., Soane L., Badea T., Shin M., Rus H.: "Tyrosine phosphorylation and activation of Janus kinase 1 and STAT3 by sublytic C5b-9 complement complex in aortic endothelial cells". *Immunopharmacology*, 1999, *42*, 187.
[34] Hoey T., Schindler U.: "STAT structure and function in signaling". *Curr. Opin. Genet. Devel.*, 1998, *8,* 582.
[35] Wery-Zennaro S., Letourneur M., David M., Bertoglio J., Pierre J.: "Binding of IL-4 to the IL-13Rα1/IL-4Rα receptor complex leads to STAT3 phosphorylation but not to its nuclear translocation". *FEBS Lett.,* 1999, *464,* 91.
[36] Zhang Q., Raghunath P.N., Xue L., Majewski M., Carpentieri D.F., Odum N. *et al.*: "Multilevel dysregulation of STAT3 activation in anaplastic lymphoma kinase-positive T/null-cell lymphoma". *J. Immunol.,* 2002, *168,* 466.
[37] Zamo A., Chiarle R., Piva R., Howes J., Fan Y., Chilosi M. *et al.*: "Anaplastic lymphoma kinase (ALK) activates STAT3 and protects hematopoietic cells from cell death". *Oncogene,* 2002, *21,* 1038.
[38] Korpelainen E.I., Karkkainen M., Gunji Y., Vikkula M., Alitalo K.: "Endothelial receptor tyrosine kinases activate the STAT signaling pathway: mutant Tie-2 causing venous malformations signals a distinct STAT activation response". *Oncogene,* 1999, *18*, 1.
[39] Binetruy-Tournaire R., Demangel C., Malavaud B., Vassy R., Rouyre S., Kraemer M. *et al.*: "Identification of a peptide blocking vascular endothelial growth factor (VEGF)-mediated angiogenesis". *EMBO J.*, 2000, *19*, 1525.

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