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Effect of thalidomide and arsenic trioxide on the release of tumor necrosis factor-α and vascular endothelial growth factor from the KG-1a human acute myelogenous leukemia cell line

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Abstract. Studies conducted in our lab have indicated that thalidomide cytotoxicity in the KG-1a human acute myelogenous leukemia (AML) cell line was enhanced by combining it with arsenic trioxide. The current investigation was conducted in order to evaluate the effect of thalidomide either alone or in combination with arsenic trioxide on the release of tumor necrosis factor-α (TNF-α) and vascular endothelial growth factor (VEGF) from this cell line in an attempt to clarify its possible cytotoxic mechanism(s). Human AML cell line KG-1a was used in this study. The cells were cultured for 48 h in the presence or absence of thalidomide (5 mg/l), and or arsenic trioxide (4 μM). The levels of TNF-α and VEGF in the supernatant were determined by ELISA. Results obtained indicate that the levels of TNF-α in the supernatant of KG-1a cell cultures incubated with thalidomide, arsenic trioxide, or combination were statistically lower than those observed in the supernatant of control cells (2.89, 5.07, 4.15 and 16.88 pg/ml, respectively). However, the levels of VEGF in the supernatant of thalidomide-treated cells were statistically higher than those in the supernatant of control cells (69.61 vs. 11.48 pg/l). Arsenic trioxide, whether alone or in combination with thalidomide, did not produce any statistically significant difference in the levels of VEGF as compared to the control or thalidomide-treated cell supernatant. These findings indicate that thalidomide and the arsenic trioxide inhibition of TNF-α production by KG-1a cells may play an important role in their cytotoxic effect.

Introduction

Studies conducted in our lab have indicated that thalidomide cytotoxicity in the KG-1a human acute myelogenous leukemia (AML) cell line was enhanced by combining it with arsenic trioxide (As_2O_3). The current investigation was conducted in order to evaluate the effect of thalidomide either alone or in combination with As_2O_3 and interleukin-2 (IL-2) on the release of tumor necrosis factor-α (TNF-α) and vascular endothelial growth factor (VEGF) from this cell line in an attempt to clarify its possible cytotoxic mechanism(s).

TNF-α is a potent pro-inflammatory cytokine produced primarily by monocytes and macrophages. Excessive or prolonged production of TNF-α played a role in inflammatory processes as well as in the pathogenesis of other human diseases (1). TNF-α appears to be particularly important in abnormal apoptosis of hematopoietic progenitors (2).

Abnormally high levels of TNF-α were associated with the pathology and symptoms of a wide variety of diseases, including erythema nodosum leprosum (ENL), acquired immune deficiency syndrome (AIDS), cancer, graft vs. host disease (GVHD), tuberculosis and malaria. Fever, weight loss and debility associated with these diseases may be due to the macrophage production of cytokines, one of which is TNF-α (3,4).

In vitro, thalidomide causes selective inhibition of TNF-α production by human monocytes triggered by lipopolysaccharides (LPS) or mycobacterial agonists (5). These effects occur at concentrations comparable with serum levels achieved *in vivo* in dosing regimens currently used, i.e., at doses of up to 400 mg/day. The level of TNF-α inhibition *in vitro* is about 40% at the clinically achievable concentration of 1 μg/ml (5).

Tumor cells with the angiogenic phenotype may overexpress one or more angiogenic proteins, mobilize an angiogenic protein from the extracellular matrix, recruit host cells that produce their own angiogenic proteins and/or down-regulate negative angiogenic regulators. The most common angiogenic proteins found in tumors are basic fibroblast growth factor (bFGF) and VEGF. Angiogenesis is a biological process whereby endothelial cells divide and migrate to form new blood vessels. Angiogenesis is a key step in various disease states, including tumor growth, invasion and metastasis (6).

Thalidomide provides its anti-angiogenesis inhibition by blocking bFGF and VEGF (7). Thalidomide inhibits angiogenesis in corneal pellets containing the angiogenic factors bFGF and VEGF, in a non-inflammatory model of angiogenesis, suggesting that thalidomide directly affects endothelial

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cells (7,8). Kruse *et al* (1998) concluded that thalidomide is a potent angiogenesis inhibitor *in vivo* (7).

Materials and methods

Human KG-1a acute myeloid leukemia cells. The KG-1a cells which are an early phenotype of human AML (American Type Culture Collection, Manassas, VA, USA) were grown in complete growth medium [Iscove's Modified Dulbeco's Medium (American Type Culture Collection) supplemented with 20% fetal bovine serum (Sigma-Aldrich, UK) and 1% penicillin-streptomycin (Gibco Invitrogen Corporation, Carlsbad, CA, USA) at 37˚C in a humidified 5% CO_2 incubator.

Treatment of human KG-1a acute myeloid leukemia cells. The KG-1a cells were cultured for 48 h in 12-well tissue culture plates, each containing complete growth medium at a concentration of $2x10^6$ cells/ml. Each well also contained a total volume of 2 ml. Thalidomide (Tocris Bioscience, Ellisville, MO, USA) was added at concentrations of 5 mg/l whether used alone or in combination with other chemotherapeutic agents. IL-2 (Proleukin®, Aldesleuken for injection) (Chiron Therapeutics, Emeryville, CA, USA) was added at a concentration of 200 IU/ml whether used alone or in combination with thalidomide and As_2O_3. As_2O_3 (Sigma-Aldrich, Inc., St. Louis, MO, USA) was added at two concentrations of 2 and 4 μM with and without 100 μM of ascorbic acid in the first flow cytometry study, and at 4 μM in the remaining studies, either alone or combined with thalidomide and IL-2. A control culture containing neither thalidomide nor IL-2 nor As_2O_3 was set up in conditions otherwise identical. The control and treated cultures were set in duplicate and incubated for 48 h at 37˚C in a humidified 5% CO_2 incubator. The incubation time was selected to allow adequate time for apoptosis and necrosis to occur in the KG-1a human myeloid leukemia cells (9).

Analysis of biologically active tumor necrosis factor. Treated and untreated KG-1a human leukemia cell line culture supernates were assayed for biologically active TNF using the Quantikine® Human TNF-α/TNFSF1A Immunoassay (R&D Systems®, Minneapolis, MN, USA), which is used for the quantitative determination of human TNF-α concentrations in cell culture supernates. Analysis was carried out as described in the Quantikine kit manual. Briefly, reagents and working standards were prepared per kit instructions. Assay diluent RD1F (50 μl) was then added to each well. Subsequently, 200 μl of the standards and samples including the controls were added to each well. The TNF-α microplate was incubated for 2 h at room temperature, after which each well was aspirated and washed with the wash buffer four times. TNF-α conjugate (200 μl) was then added to each well and the wells were incubated for another hour. After that the wells were aspirated and washed again four times with the washing buffer. This was followed by the addition of 200 μl of substrate solution to each well, and the microplate was incubated for 20 min away from light. Stop solution (50 μl) was then added to each well. The optical density of each well was determined within 30 min, using a microplate reader (Power Wave X 340, Bio-Tek® Instruments, Inc., Winooski, VT, USA) set to 450 nm (λ correction at 570 nm) (10).

Measurements of the vascular endothelial growth factor. Treated and untreated KG-1a human leukemia cell line culture supernates were assayed to measure the concentration of VEGF using the Quantikine Human VEGF Immunoassay (R&D Systems), which is used for the quantitative determination of human VEGF concentrations in cell culture supernates. Analysis was carried out as described in the Quantikine kit manual. This assay employs the quantitative sandwich enzyme immunoassay technique. Briefly, reagents and working standards were prepared per kit instructions. Assay diluent RD1W (50 μl) was added to each well. Subsequently, 200 μl of the standards and samples, including the controls, were added to each well. The VEGF microplate was incubated for 2 h at room temperature, after which each well was aspirated and washed with the wash buffer three times. VEGF conjugate (200 μl) was then added to each well and wells were incubated for 2 h. Following this incubation, wells were aspirated and washed again three times with the washing buffer. Substrate solution (200 μl) was added to each well, and the microplate was incubated for 20 min away from light. Stop solution (50 μl) was then added to each well. The optical density of each well was determined within 30 min, using a microplate reader (Power Wave X 340, Bio-Tek Instruments, Inc.) set to 450 nm (λ correction at 570 nm) (11).

Statistical analysis. Results were subjected to one-way ANOVA. Statistical significant differences between means was set at $p<0.05$.

Results

Human tumor necrosis factor-α immunoassay (ELISA). The objective of ELISA, which employs the quantitative sandwich enzyme immunoassay technique, is to evaluate the effect of thalidomide, IL-2, and As_2O_3 or their combination on the level of TNF-α in the cell culture supernate of the KG-1a human leukemia cell line. This evaluation was conducted in an attempt to elucidate the possible mechanism of thalidomide cytotoxicity as well as the enhancement mechanism of As_2O_3 on this cytotoxicity. Fig. 1 shows the results obtained. The following levels of TNF-α were found in the supernatant of the various treatment groups: i) Control, 16.89±4.43 pg/ml; ii) IL-2, 2.64±0.48 pg/ml; iii) As_2O_3, 5.07±0.69 pg/ml; iv) IL-2 and As_2O_3, 2.89±0.91 pg/ml; v) thalidomide, 2.89±0.25 pg/ml; vi) thalidomide and IL-2, 3.98±0.30 pg/ml; vii) thalidomide and As_2O_3, 4.15±0.25 pg/ml and viii) thalidomide, IL-2 and As_2O_3, 2.96±1.13 pg/ml.

From the results, it is evident that TNF-α levels significantly decreased in KG-1a cells incubated with the chemotherapeutic agents alone or in combination.

Human vascular endothelial growth factor immunoassay (ELISA). ELISA was used for the quantitative determination of human VEGF concentration in the cell culture supernate of the KG-1a human leukemia cell line following the application of the different chemotherapeutic agents. This determination was performed to evaluate the anti-angiogenic effect of thalidomide when used alone or in combination with the remaining chemotherapeutic agents. Fig. 2 shows the results obtained. The VEGF levels observed in the supernatant of the various

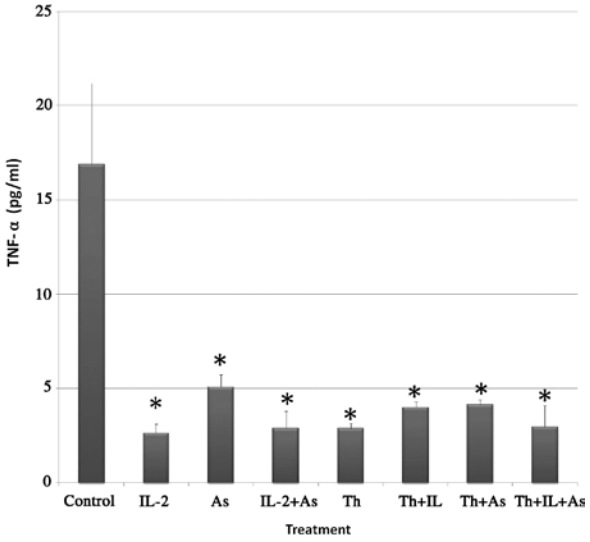

Figure 1. Tumor necrosis factor α (TNF-α) in the supernatant of KG-1a cells incubated with thalidomide (Th) (5 mg/l), arsenic trioxide (As) (4 μM), interleukin-2 (IL-2) (200 IU/ml) alone or in combination. Bars represent mean ± SE. *Significantly different from control ($p<0.05$).

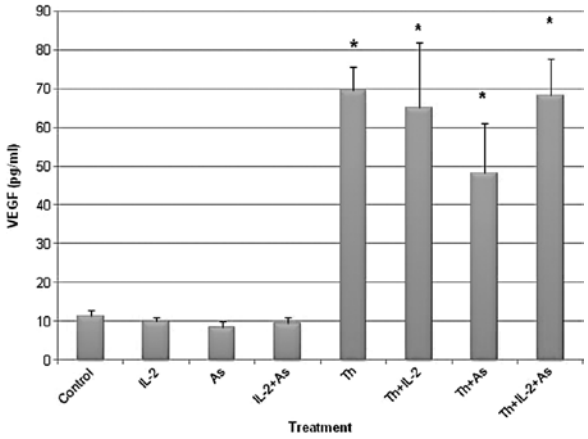

Figure 2. Vascular endothelial growth factor (VEGF) in the supernatant of KG-1a cells incubated with thalidomide (Th) (5 mg/l), arsenic trioxide (As) (4 μM), interleukin-2 (IL-2) (200 IU/ml) alone or in combination. Bars represent mean ± SE. *Significantly different from control ($p<0.05$).

treatment groups included: i) Control, 11.42±1.43 pg/ml; ii) IL-2, 9.99±1.15 pg/ml; iii) As_2O_3, 8.51±1.48 pg/ml; iv) IL-2 and As_2O_3, 9.35±1.78 pg/ml; v) thalidomide, 69.61±5.99 pg/ml; vi) thalidomide and IL-2, 65.07±16.83 pg/ml; vii) thalidomide and As_2O_3, 48.13±12.82 pg/ml and viii) thalidomide and IL-2 and As_2O_3, 68.19±9.57 pg/ml.

The results showed that thalidomide treatment increased the VEGF levels in contrast to IL-2 and As_2O_3 which did not affect the VEGF levels.

Discussion

This study was conducted as a follow-up to our previous study whose purpose was to evaluate the efficacy of thalidomide in the management of AML and to test the possibility of enhancing its cytotoxicity by combining it with other chemotherapeutic agents such as IL-2 and As_2O_3. The variant subline KG-1a of the human acute myelogenous leukemia cell line KG-1 was used as a test model in both the previous and current studies.

The present study hypothesized that the enhancement of cytotoxicity induced by thalidomide is due to the effects on TNF-α and VEGF. This hypothesis stems from the observation that angiogenesis increases tumor growth via perfusion as well as the paracrine production of growth factors by endothelial cells or their release by macrophage and other host cells (12). The most common angiogenic proteins noted in tumors are VEGF and bFGF. VEGF is a potent mitogen for vascular endothelial cells that has been associated with angiogenesis, growth, dissemination, metastasis, and poor outcome in solid tumors (13). Fiedler *et al* (14) found VEGF transcription in 23 of 33 (69%) patients with AML. Leukemic cell cultures from 24 of these patients produced significantly high VEGF levels (14). In addition, TNF-α is a potent pro-inflammatory cytokine produced primarily by monocytes and macrophages. Excessive or prolonged production of TNF-α played a role in inflammatory processes as well as in the pathogenesis of other human diseases (1). TNF-α appears to be particularly important in abnormal apoptosis of hematopoietic progenitors (2). Consequently, we attempted to clarify the role of TNF-α and VEGF in cytotoxicity induced by thalidomide, As_2O_3 and IL-2.

The results obtained in the current study indicate that thalidomide pretreatment decreased the levels of TNF-α in the supernates from 16.9 pg/ml in the control to 2.9 pg/ml. This effect was also detected when cells were pretreated with either IL-2 or As_2O_3 (2.64 and 5.07 pg/ml, respectively). When thalidomide was used concurrently with As_2O_3 and IL-2 the levels of TNF-α were also significantly lowered as compared to the control (4.15 and 3.98 pg/ml, respectively).

The results are consistent with other studies which showed that thalidomide inhibits TNF-α (15-19). Thalidomide inhibits the synthesis of TNF-α *in vitro* and *in vivo*. Abnormally high levels of TNF-α have been associated with the pathology and symptoms of a wide variety of diseases, including ENL, AIDS, cancer, GVHD, tuberculosis and malaria. Fever, weight loss, and debility associated with these diseases may be due to the macrophage production of cytokines, one of which is TNF-α (3,4). Animal models used to investigate the activity of TNF-α have shown that infusion of purified recombinant TNF-α reproduces the characteristics of septic shock syndrome, including severe hypotension, lactic acidosis, third-space fluid loss, and tissue injury (4). Endothelial cell damage, disturbance of lipid metabolism and interleukin-1 (IL-1) release also occur. Furthermore, chronic sublethal injection of TNF-α produces cachexia in rats (4).

In vitro, thalidomide causes selective inhibition of TNF-α production by human monocytes triggered by LPS or mycobacterial agonists (5). These effects occur at concentrations comparable with serum levels achieved *in vivo* in dosing regimens currently used, i.e., at doses of up to 400 mg/day. The level of TNF-α inhibition *in vitro* is about 40% at the clinically achievable concentration of 1 μg/ml (5). Thalidomide does not directly influence the production of other cytokines (IL-1β, IL-6 and GM-CSF) that may be important in conditions where suppression of TNF-α is desirable, but immunity must otherwise remain intact (16).

Thalidomide results in decreased TNF-α production by accelerating the degradation of mRNA encoding the protein (16). This mechanism is different from the mechanism of action proposed for pentoxifylline or corticosteroids, which suppress LPS-induced TNF-α RNA transcription and translation, respectively. Notably, the results are consistent with other studies which showed that thalidomide inhibits TNF-α (15-19). The ability of thalidomide to reduce elevated levels of TNF-α makes the drug of potential therapeutic importance in any disease state where high TNF-α levels cause primary problems or secondary complications.

However, results obtained from the VEGF immunoassay in the current study indicate that thalidomide pretreatment increases VEGF levels in the supernates from 11.42 pg/ml in the control to 69.61 pg/ml. This effect was also detected when cells were pretreated with thalidomide combined with IL-2, As_2O_3 or both (65.07, 48.13 and 68.19 pg/ml, respectively). Moreover, when cells were pretreated with either IL-2 or As_2O_3 VEGF levels were not different from the control (9.99 and 8.51 pg/ml, respectively compared to 11.42 pg/ml for the control). These results confirm that thalidomide has an important role in angiogenesis. Angiogenesis is a biological process in which endothelial cells divide and migrate to form new blood vessels. Anti-angiogenesis involves therapy against biochemical targets on the neovasculature, inhibiting proliferation and blood vessel formation. Angiogenesis is therefore significant in various disease states, including tumor growth, invasion and metastasis (6).

Thalidomide provides anti-angiogenesis inhibition by blocking bFGF and VEGF (7). Thalidomide inhibits angiogenesis in corneal pellets that contain angiogenic factors, bFGF and VEGF, in a non-inflammatory model of angiogenesis. Thus, thalidomide may directly affect endothelial cells (7,8). Kruse *et al* concluded that thalidomide is a potent angiogenesis inhibitor *in vivo*.

The increase of VEGF in our study was due to the release of growth-promoting substances, including VEGF and bFGF, by the surviving KG-1a leukemic cells. This increase of VEGF levels likely reflects an autologous protective mechanism or mutation by the surviving malignant leukemic cells to increase their resistance to subsequent chemotherapy. Another comparable study that supports our explanation regarding the increase of VEGF was conducted by Brieger *et al* (20). These authors reported on the time- and dose-dependent release of VEGF and bFGF from squamous cell carcinoma (SCC) cells in cell culture after irradiation. These authors also demonstrated that these factors are possible protectors from the irradiation-induced cell death of cancer cells. The hypothesis of autologous protection of tumor cells from radiation-induced cell death by secreted factors is supported by several studies. Shintani *et al* (21) reported increased VEGF-levels in oral SCC in the case of non-responders to radiotherapy. Similar data were noted by Koukourakis *et al* (22) in head and neck SCC. Geng *et al* (23) showed that inhibition of VEGF-signaling results in the reversal of tumor resistance to radiotherapy. Taken together, high VEGF levels and consecutive activation of the VEGF-receptor and downstream signal transduction pathways may result in a direct cytoprotection of tumor cells or in the protection of vessels and increased neoangiogenesis. Therefore, ionizing radiation promotes tumor cell survival, as well as desired effects such as the induction of apoptosis and growth arrest (20).

In our study, the effects of thalidomide were comparable to radiotherapy since the former showed considerable cytotoxic effect as evidenced by flow cytometry studies. Thalidomide also exhibited autologous protection of KG-1a leukemia cells by increasing secretion of VEGF from the cells that survived.

In conclusion, this study showed that thalidomide, As_2O_3 and IL-2 decreased the level of tumor necrosis-α in the cell supernatant in a treated KG-1a human acute myelogenous leukemia cell line. On the other hand, it was shown that thalidomide increased the release of vascular endothelial growth factor in the cell supernatant. This increase suggests an autologous protective mechanism or mutation by the surviving malignant leukemic cells to increase their resistance to subsequent chemotherapy.

Acknowledgements

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