Induction of Th1 chemokine secretion in dermal fibroblasts by vanadium pentoxide

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Abstract. Vanadium is a soft, silvery-grey metal with a number of different oxidation states. The most common commercial form of vanadium is vanadium pentoxide (V_2O_5). All vanadium compounds are considered toxic. An increase in skin rashes has been observed in certain vanadium workers, including the development of atopic dermatitis. However, to the best of our knowledge, no prior *in vivo* or *in vitro* studies have evaluated the effect of vanadium exposure in human dermal fibroblasts. The present study evaluated the effect of V_2O_5 on proliferation and chemokine secretion in dermal fibroblasts. The results revealed that V_2O_5 had no significant effect on the viability or proliferation of fibroblasts, however it was able to induce the secretion of T-helper (Th)1 chemokines from dermal fibroblasts, synergistically increasing the effect of important Th1 cytokines, including interferon-γ and tumor necrosis factor-α. Through these processes, V_2O_5 may lead to the induction and perpetuation of an inflammatory reaction in dermal tissue. The induction and perpetuation of inflammation in the dermis and the variety of involved candidate genes may be at the base of V_2O_5-induced effects following occupational and environmental exposures. Further studies are necessary to evaluate dermal integrity and manifestations in subjects who are occupationally exposed, or living in polluted areas.

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Introduction

Vanadium is a soft, silvery-grey metal, which exists in a number of different oxidation states (-1, 0, +2, +3, +4 and +5); vanadium pentoxide (V_2O_5) is the most common commercial form, and most of the studies on toxicity have been conducted on vanadium pentoxide, as it is the primary form found in industrial exposure situations (1). All vanadium compounds are considered toxic. The Occupational Safety and Health Administration (Bellevue, WA, USA) have set an exposure limit for the workplace (considering an 8 h workday, and a 40 h work week), of 0.05 mg/m^3 for V_2O_5 dust and 0.1 mg/m^3 for V_2O_5 fumes (2).

The exposure dose of vanadium that is considered life-threatening is 35 mg/m^3 [as determined by the National Institute for Occupational Safety and Health (NIOSH; Washington, DC, USA)], which could cause serious and perpetuating health issues, including death (2). The respiratory system is the most vulnerable to vanadium toxicity, while the effect on the gastrointestinal system is minimal due to the low gut absorption rate (3-5). However, quantitative data are not sufficient to obtain a chronic or subchronic inhalation reference dose.

In rat models, the effects resulting from an inhaled or oral vanadium were evaluated in the sera (6,7), nervous tissue (8), liver (9) and other types of tissue (kidney, gut, lungs) development (10). In vanadium workers (NIOSH 1983) increases in skin rashes and atopic dermatitis have been recorded. To the best of our knowledge, no prior *in vivo* or *in vitro* studies have been conducted to evaluate the effect of vanadium exposure on dermal fibroblasts. Here, we evaluated the effect of V_2O_5 on the proliferation and chemokine secretion profiles of dermal fibroblasts.

Materials and methods

Fibroblast cell cultures. Dermal fibroblasts were obtained from 6 patients who underwent surgery for thyroid nodular goiter (discarded dermal material was used). The local Ethics Committee of the University of Pisa approved the study protocol, and all subjects provided informed consent.

As previously described, tissue explants from the derma were minced and placed in culture dishes, to allow the fibroblasts to proliferate (11). Fibroblasts were propagated in Medium 199 containing 20% FBS (Gibco; Invitrogen, Ltd., Paisley, UK), gentamycin (20 μg/ml) and penicillin (100 U/ml), in a 37˚C humidified incubator with 5% of CO_2. Cells were subsequently maintained in medium 199 containing 10% FBS (and antibiotics) (12).

Cell viability and proliferation assay. The evaluation of cell proliferation and viability was conducted using a WST-1 assay (Roche Diagnostics, Almere, The Netherlands), which uses MTT (13,14). Fibroblasts were seeded (35,000 cells/ml, in a final volume of 100 μl) into 96-well plates. The effect V_2O_5 on fibroblast proliferation was determined following exposure of the cells for 24 h to increasing concentrations of V_2O_5 (1, 10 and 100 nM). Cells were then plated and treated for 24 h with V_2O_5, or with its vehicle alone; all experiments were performed in triplicate for each cell preparation.

Proliferation assay: cell counting. The cell counting assay was also used to assess fibroblast proliferation (13,14).

Chemokines secretion assays. Chemokine (C-X-C motif) ligand (CXCL)9 and CXCL10 secretion assays were performed by seeding 30,000 cells/ml into 96-well plates, with a final volume of 100 μl/well, in growth medium that was removed after 24 h. Cells were subsequently washed in PBS, then incubated (24 h) in phenol red and serum-free medium with interferon (IFN)-γ (R&D Systems, Minneapolis, MN, USA; 500; 1,000; 5,000; 10,000 IU/ml), and/or 10 ng/ml tumor necrosis factor (TNF)-α (R&D Systems) (11). Preliminary experiments were conducted to select the TNF-α concentration, in order to obtain the highest secretion rate. The supernatants were collected after 24 h, then frozen at -20˚C until use in the chemokine assay.

To understand the effect of V_2O_5 on the chemokine secretion induced by IFN-γ, cells were treated for 24 h with increasing concentrations of V_2O_5 (1, 10 and 100 nM), in the presence or absence of IFN-γ (1,000 IU/ml), and/or TNF-α (10 ng/ml). An ELISA was used to measure the CXCL9 and CXCL10 levels in the supernatants. The experiments were performed three times for each different cell preparation.

ELISA for CXCL9 and CXCL10. CXCL9 and CXCL10 were assessed in the supernatants obtained from cell cultures, using commercially available kits (R&D Systems). The minimum (mean) detectable doses were 1.5 and 1.2 pg/ml for CXCL9 and CXCL10, respectively. The intra- and inter-assay coefficients of variation were 3.5 and 6.4% respectively, for CXCL9, and 4.5 and 7.3% respectively, for CXCL10. Quality control pools of normal, low and high concentrations were also included in each assay.

Data analysis. For normally distributed variables, values are given as the mean ± SD in text, and in figures, otherwise as the median and interquartile range. Mean group values were compared using one-way analysis of variance (ANOVA) for variables normally distributed variables, or by using the Kruskal-Wallis test or Mann-Whitney U test. Proportions were compared using the Chi-square test. In addition, the Bonferroni-Dunn test was used for the post hoc comparison of normally distributed variables.

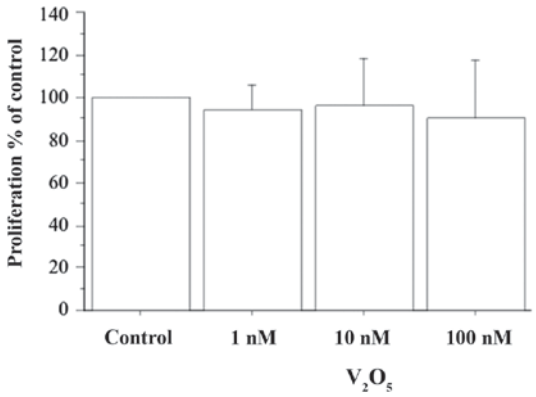

Figure 1. The WST-1 cell viability and proliferation assay showed that V_2O_5 (1, 10 and 100 nM) does not alter the viability or proliferation of dermal fibroblasts (mean group values were compared using one-way analysis of variance; the Bonferroni-Dunn test was used for post hoc comparisons; $P>0.05$ for all comparisons). V_2O_5, vanadium pentoxide.

Results

Cell proliferation. The WST-1 cell viability and proliferation assay showed that V_2O_5 (1, 10 and 100 nM) did not alter the viability or proliferation of dermal fibroblasts (Fig. 1). These results were confirmed by a cell counting assay (data not presented).

CXCL9. CXCL9 was not detectable in the supernatants gathered from primary fibroblast samples, whereas its concentration was elevated following IFN-γ dose-dependent induction (0, 75±31, 141±29, 210±35 and 297±74 pg/ml for IFN-γ 0; 500; 1,000; 5,000 and 10,000 IU/ml, respectively; $P<0.001$, ANOVA). TNF-α alone had no significant impact on CXCL9, which remained undetectable, whereas IFN-γ plus TNF-α exhibited a synergistic effect on the CXCL9 release (CXCL9, 11,154±1,673 vs. 151±42 pg/ml with IFN-γ alone; $P<0.0001$, ANOVA).

CXCL9 release was dose-dependently stimulated ($P<0.0001$, ANOVA) when fibroblasts were treated with increasing V_2O_5 concentrations (1, 10 and 100 nM) (Fig. 2A). Following the treatment of fibroblasts with V_2O_5 (1, 10 and 100 nM), together with TNF-α, CXCL9 secretion was not significantly changed with respect to V_2O_5 alone (data not presented). Treating fibroblasts with 100 nM V_2O_5 plus IFN-γ induced a synergistic increase in CXCL9 release ($P<0.0001$, ANOVA) (Fig. 3A). When fibroblasts were treated with V_2O_5 (100 nM), together with IFN-γ and TNF-α stimulation, CXCL9 release was synergistically increased ($P<0.0001$, ANOVA) (Fig. 4A).

CXCL10. CXCL10 was also not detectable in the supernatants obtained from primary fibroblast cultures under basal conditions. IFN-γ induced CXCL10 secretion dose-dependently (0, 34±18, 107±42, 187±32 and 272±76 pg/ml, respectively, for IFN-γ 0; 500; 1,000; 5,000; 10,000 IU/ml; ANOVA, $P<0.001$). TNF-α alone did not have a significant impact on CXCL10 secretion, whereas IFN-γ plus TNF-α exhibited a synergistic

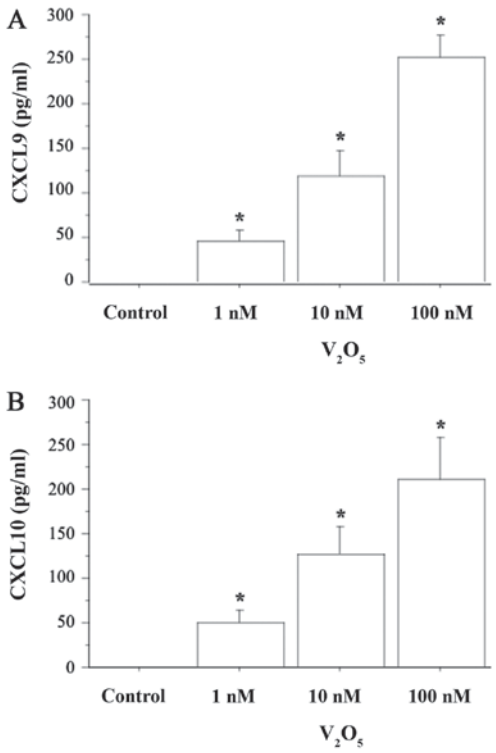

Figure 2. Following the treatment of dermal fibroblasts with V_2O_5 (1, 10 and 100 nM), (A) CXCL9 and (B) CXCL10 secretion was dose-dependently stimulated (P<0.0001 by one-way analysis of variance). Bonferroni-Dunn test was used for post hoc comparisons. *P<0.05 vs. the control. V_2O_5, vanadium pentoxide; CXCL, chemokine (C-X-C motif) ligand.

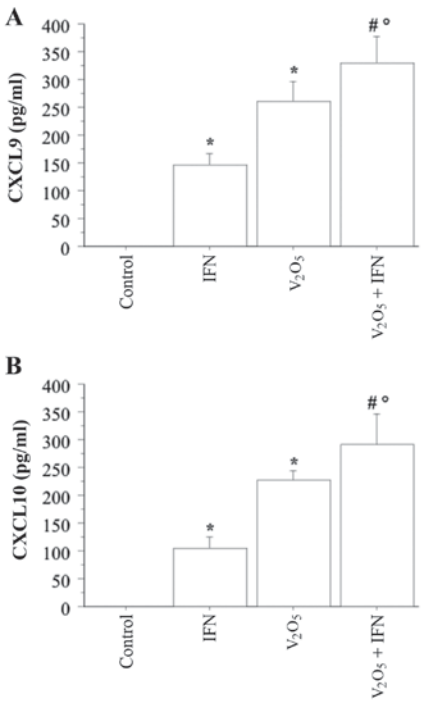

Figure 3. Treating dermal fibroblasts with V_2O_5 (100 nM), together with IFN-γ, synergistically increased (A) CXCL9 and (B) CXCL10 release (P<0.0001 by one-way analysis of variance). Bonferroni-Dunn test was used for post hoc comparisons. *P<0.05 vs. the control; #P<0.05 vs. IFN; °P<0.05 vs. V_2O_5. V_2O_5, vanadium pentoxide; CXCL, chemokine (C-X-C motif) ligand; IFN, interferon.

effect on CXCL10 secretion (3,043±234 vs. 117±27 pg/ml with IFN-γ alone; P<0.0001, ANOVA).

CXCL10 release was dose-dependently stimulated (P<0.0001, ANOVA) when fibroblasts were treated with increasing V_2O_5 concentrations (1, 10 and 100 nM) (Fig. 2B). Following the treatment of fibroblasts with V_2O_5 (1, 10 and 100 nM), and together with TNF-α, CXCL10 secretion was not significantly changed with respect to V_2O_5 alone (data not presented).

Treating fibroblasts with 100 nM V_2O_5 *plus* IFN-γ caused a synergistic increase in CXCL10 release (P<0.0001, ANOVA) (Fig. 3B). When fibroblasts were treated with V_2O_5 (100 nM) together with IFN-γ and TNF-α stimulation, CXCL10 release was also synergistically increased (P<0.0001, ANOVA) (Fig. 4B).

Discussion

The results of the present study demonstrated that V_2O_5 could promote IFN-γ-dependent chemokine secretion in dermal fibroblasts, without altering their cell proliferation and viability. In addition, our results confirmed that IFN-γ and TNF-α stimulate CXCL9 and CXCL10 secretion, as hypothesized (11,15). It is notable that V_2O_5 was able to synergize with IFN-γ and TNF-α, further increasing chemokines secretion.

These results are concordant with the hypothesis that V_2O_5 is able to induce and perpetuate inflammation in the dermal tissues, evolving from a predominant T-helper (Th)1 immune response (13). IFN-γ-inducible C-X-C chemokines are secreted by several types of mammalian cells, including fibroblasts,

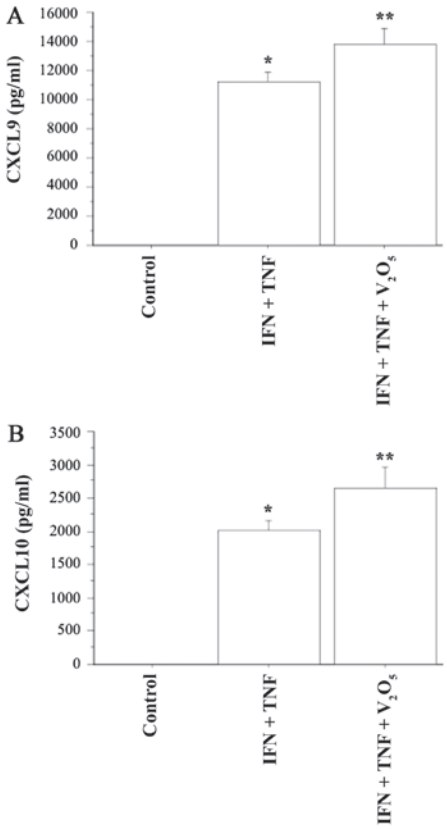

Figure 4. Treating dermal fibroblasts with V_2O_5 (100 nM), together with IFN-γ plus TNF-α, synergistically increased (A) CXCL9 and (B) CXCL10 secretion (P<0.0001 by one-way analysis of variance). Bonferroni-Dunn test was used for post hoc comparisons. *P<0.05 vs. the control; **P<0.05 vs. IFN+TNF. V_2O_5, vanadium pentoxide; CXCL, chemokine (C-X-C motif) ligand; IFN, interferon; TNF, tumor necrosis factor.

thyrocytes, islet cells, colon epithelial cells and endothelial cells, among others (11,13-21). These cell types are unable to produce these chemokines under basal conditions; they are induced following stimulation by IFN-γ (alone or in combination with TNF-α), a cytokine that is produced by Th1-activated lymphocytes in several autoimmune diseases, including in the thyroid in Graves' disease, and in autoimmune thyroiditis. It has been hypothesized that this process can be involved in the initiation and/or the perpetuation of various autoimmune disorders (11,13-21), and that it may also be applied to the thyroid.

Our results are concordant with those of other studies in different cell types. V_2O_5 exposure is a cause of occupational bronchitis; an *in vitro* study was conducted to evaluate the gene expression profiles of human lung fibroblasts following V_2O_5 exposure, in order to identify genes that might play a role in the bronchial inflammation, repair and fibrosis during the pathogenesis of bronchitis. A dozen genes are overexpressed by V_2O_5, including *CXCL9* and *CXCL10* (1). A further study reported that fibroblasts responded to vanadium oxidative stress by producing IFN-β and activating STAT-1, which lead to increased CXCL10 levels (22), thus serving a role in the innate immune response.

It is notable that vanadium is able to increase chemokine secretion in the dose range of 1-100 nM. Since the normal blood levels of vanadium range from 0.45-18.4 nM, 100 nM could be noted as a dose that might mimic an abnormally high exposure (23). Thus, we could hypothesize that V_2O_5 in this concentration range is able to induce an inflammatory reaction in dermal tissues, prompting the appearance of skin rashes or atopic dermatitis.

Moreover, it has been shown that exposure of human skin fibroblasts to vanadate causes DNA strand breaks at relevant concentration of 1 μM (24). In the present study we have considered lower concentrations (1, 10 and 100 nM), that did not alter the viability or proliferation of dermal fibroblasts.

In conclusion, the results of our study showed that V_2O_5 is able to induce Th1 chemokine secretion in dermal tissues, and that it can synergize with important Th1 cytokines (such as IFN-γ and TNF-α), leading to the induction and perpetuation of inflammation in the dermis. Moreover, different genes are overexpressed by V_2O_5, including *CXCL9* and *CXCL10*, that appear to have important functions in inflammation, fibrosis and repair. To the best of our knowledge, no prior study has evaluated the immune modulatory effects of vanadium in dermal fibroblasts; therefore, our results could be important for evaluating the pathogenesis of clinical dermatological manifestations of vanadium exposure in humans. The induction and perpetuation of inflammation in the dermis and the variety of involved candidate genes could be at the basis of V_2O_5-induced effects after occupational and environmental exposures. Additional studies are required to assess dermal integrity, as well as the manifestations of toxicity in subjects who are occupationally exposed, or are living in polluted areas.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

PF, RF, AC, AA and SMF made substantial contributions to the conception and design of the study and to the acquisition of the data. GE, FR, AP, GG, GF and SB analysed the data. PF, AA and SMF. have been involved in drafting the manuscript. AA critically revised the manuscript for important intellectual content.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors confirm that they have no competing interests.

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