CXCL8 and CXCL11 chemokine secretion in dermal fibroblasts is differentially modulated by vanadium pentoxide

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Abstract. An increase in skin rashes or atopic dermatitis has been observed in individuals working with vanadium. However, to the best of our knowledge no in vivo or in vitro studies have evaluated the effect of exposure to vanadium in dermal fibroblasts. Cells viability and proliferation were assessed by WST-1 assay, cells were treated with increasing concentrations of V2O5 (1, 10 and 100 nM). CXCL8 and CXCL11 concentrations were measured in the supernatants using an ELISA assay. V2O5 was not observed as having a significant effect on dermal fibroblast's viability and proliferation. However, it was revealed that V2O5 was able to induce the secretion of CXCL8 and CXCL11 chemokines into dermal fibroblasts. V2O5 synergistically increased the effect of interferon (IFN)γ on CXCL11 secretion. In addition, V2O5 synergistically increased the effect of the tumor necrosis factor α on CXCL8 secretion and abolished the inhibitory effect of IFNγ. V2O5 induction of CXCL8 and CXCL11 chemokines may lead to the appearance and perpetuation of an inflammatory reaction into the dermal tissue. Further studies are required to evaluate dermal integrity and manifestations in subjects occupationally exposed, or living in polluted areas.

Introduction

Vanadium is a grey metal that exists in different states of oxidation (ranging from -1 to +5) of which vanadium pentoxide (V2O5) is the most usual form.

All vanadium compounds have been considered toxic. The exposure limit to V2O5 dust and fumes in workplace air (8 h work day/40 h work week) has been fixed by the Occupational Safety and Health Administration in 0.05 and 0.1 mg/m3, respectively (1).

The National Institute for Occupational Safety and Health (NIOSH) sets to 35 mg/m3 the dose of vanadium exposure that may cause seriously health issues up to death (1).

Toxic effects of vanadium are reflected mainly on respiratory system, while the effect on the gastrointestinal system is less relevant because of the minimal gut absorption rate of the substance (2-4). Unfortunately, no sufficient data are available in order to determine the reference range of a subchronic or chronic inhaled dose.

Studies conducted on rat models showed the toxic effects (resulted from an oral, or inhaled, vanadium exposures) on serum parameters (5,6), liver (7), nervous (8) and other tissues development (9).

Vanadium workers (NIOSH 1983) showed an increased prevalence of skin rashes, such as atopic dermatitis.

Until now no in vivo, or in vitro, studies were carried out to evaluate the effect of exposure to vanadium in dermal fibroblasts.

Here, we evaluate the effect of V2O5 on viability and proliferation, and secretion of chemokine (C-X-C motif) ligand (CXCL)8, or CXCL11 [an interferon (IFN)γ dependent chemokine, of the same class of CXCL9, and CXCL10] in dermal human fibroblasts.

Materials and methods

Fibroblast cell cultures. We have obtained fibroblasts from derma of six patients who underwent an operation for thyroid nodular goiter (discard dermal material; all females, age range 57-76 years, euthyroid, without other disorders or diseases, and not treated with any kind of drugs).

Involved subjects gave their informed consent and the study was approved by the University of Pisa (Pisa, Italy) Ethics Committee. Tissue explants were firstly minced and then placed in culture dishes, allowing the fibroblasts proliferation (as previously described) (10). Fibroblasts were propagated in 199 medium [with 20% FBS (Gibco; Thermo Fisher Scientific,
Waltham, MA, USA), gentamycin (20 µg/ml), penicillin (100 U/ml), in a 37°C humidified incubator with 5% CO₂ and maintained subsequently in a 199 medium with 10% FBS (and antibiotics) (11). The cells were all used at the 4th passage, and were tested for purity by immunocytochemistry (12).

Proliferation and viability. We have done the WST-1 (Roche Diagnostics, Almere, The Netherlands) assay (that uses 3-[4,5-dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide, in the MTT assay) to evaluate cell viability and proliferation (13-16).

Firstly fibroblasts were seeded in each well of 96-well plates at a concentration of 35,000 cells/ml (in a final volume of 100 µl).

Subsequently V₅O₃ effect on fibroblasts viability and proliferation was determined exposing cells for 24 h with increased concentrations of the compound (1, 10, 100 nM).

Fibroblasts were plated and treated with V₅O₃ or with its vehicle alone (for 24 h), performing all experiments in triplicate for each cell preparation.

As the cell viability and proliferation WST-1 assay may have limitations on evaluating cellular proliferation (17), fibroblasts proliferation was determined also by cell number counting (13-16).

Chemokine secretion assay and ELISA. To perform the CXCL8 and CXCL11 secretion assays, 30,000 cells/ml were seeded in 96-well plates, in a final volume of 100 µl per well, in growth medium, that was removed after 24 h. After cells were washed in PBS, and incubated (24 h) in phenol red and serum-free medium containing IFNγ (500, 1,000, 5,000, 10,000 IU/ml) and/or 10 ng/ml TNFα (all R&D Systems, Minneapolis, MN, USA), alone or in combination (10). The TNFα concentration to obtain the highest secretion was selected in preliminary experiments. After 1 day the supernatants were collected and then kept frozen at -20°C (until chemokine assay).

We treated fibroblasts, for 24 h, with increasing concentrations of V₅O₃ (1, 10, 100 nM), in presence/absence of IFNγ (1,000 IU/ml), and/or TNFα (10 ng/ml), in order to evaluate the effect of V₅O₃ on the chemokine secretion induced by IFNγ.

CXCL8 and CXCL11 concentrations were measured in the supernatants using the ELISA assay. The experiments were carried out three times, for each different cell preparation.

Chemokines levels were measured in culture supernatants, using commercially kits (R&D Systems). The mean minimum detectable dose was 2.7 pg/ml for CXCL8 and 3.2 pg/ml for CXCL11; the intra- and inter-assay coefficients of variation were 3.5 and 6.5% for CXCL8, 4.7 and 8.5% for CXCL11. Quality control pools of low, normal, or high concentration for each parameter were included in each assay.

Statistical analysis. For normally distributed variables values are given in text as mean (±SD), or mean (±SEM) in figures, otherwise as median [and interquartile range]. Mean group values are compared by one-way analysis of variance (ANOVA) for variables normally distributed, or with the Kruskal-Wallis test, or Mann-Whitney U test. Proportions are compared by the Chi-Square. We have used the Bonferroni-Dunn test for post hoc comparison of normally distributed variables.

Results

Cell proliferation of dermal fibroblasts. Cell counting shows that V₅O₃ (1, 10, 100 nM) does not change viability or proliferation of dermal fibroblasts (Fig. 1). The results of WST-1 assay in dermal fibroblasts with V₅O₃ (1, 10, 100 nM) confirmed the cell counting data: with V₅O₃ 1 nM it was 99% with respect to the control; with V₅O₃ 10 nM it was 97% with respect to the control; and with V₅O₃ 100 nM it was 98% with respect to the control.

Fibroblast secretion of CXCL8. In basal conditions, the secretion of CXCL8 (range, 51-213 pg/ml) was measured in all preparations of cultured dermal fibroblasts (Fig. 2).

CXCL8 secretion increased in a dose-dependent manner using different concentrations of TNFα (1, 5, 10 ng/ml), with the highest response reached with 10 ng/ml TNFα (basal 156±46 pg/ml vs. TNFα 1154±321 pg/ml; P<0.01) (Fig. 2).

The basal CXCL8 secretion was significantly inhibited by IFNγ in a dose-dependent manner (CXCL8: 84±37, 34±25 pg/ml; respectively, with IFNγ 500 or 1000 IU/ml; ANOVA, P<0.05), while TNFα alone (10 ng/ml) significantly stimulated the CXCL8 secretion (P<0.01) (Fig. 3). Combining IFNγ with TNFα led to a significant reversal of the stimulating effect of TNFα (TNFα+IFNγ 661±176 pg/ml vs. TNFα 1154±321 pg/ml; P<0.05) (Fig. 3). However, the stimulating effect of TNFα on the secretion of CXCL8 was not completely reversed by IFNγ, because the concentration of this chemokine was still significantly higher than in basal conditions (TNFα+IFNγ vs. basal; P<0.01).

When fibroblasts were treated with increased V₅O₃ concentrations (1, 10, 100 nM) the CXCL8 release was dose-dependently stimulated (P<0.0001, by ANOVA) (Fig. 4).

When treating dermal fibroblasts with V₅O₃ (100 nM), together with IFNγ, CXCL8 release was not significantly changed with respect to the basal condition, and IFNγ suppressed the V₅O₃ stimulating effect, but it stills increased it compared to IFNγ alone (Fig. 5).

V₅O₃ (100 nM) plus TNFα elicited a synergistic effect on CXCL8 secretion (P<0.0001, by ANOVA), compared to TNFα alone (Fig. 6).

The CXCL8 release synergistically increased (P<0.0001, by ANOVA), when fibroblasts were treated with V₅O₃ (100 nM) with the combination of IFNγ and TNFα, abolishing the inhibitory effect of IFNγ (Fig. 7).

Fibroblast secretion of CXCL11. CXCL11 release was induced by IFNγ in a dose-dependent manner (CXCL11: 0, 31±17, 87±35, 123±47, 187±52 pg/ml; respectively, with IFNγ 0, 500, 1,000, 5,000, 10,000 IU/ml; ANOVA, P<0.001).

TNFα alone had no effect (chemokine remaining undetectable), while the combination of IFNγ and TNFα had a significant synergistic effect on the CXCL11 secretion (CXCL11, 1724±252 vs. 87±35 pg/ml with IFNγ alone, ANOVA, P<0.0001).

When fibroblasts were treated with increased V₅O₃ concentrations (1, 10, 100 nM) the CXCL11 release was dose-dependently stimulated (ANOVA, P<0.0001) (Fig. 8).

CXCL11 release was not significantly changed treating cells with V₅O₃ (100 nM), together with TNFα, with respect to V₅O₃ alone (data not shown).
When treating fibroblasts with V$_2$O$_5$ (100 nM), plus IFN$_\gamma$, CXCL11 release synergistically increased (P<0.0001, by ANOVA), compared to both IFN or V$_2$O$_5$ alone (Fig. 9).

Our results demonstrate that V$_2$O$_5$ stimulates the secretion of the CXCL8 chemokine, and of the IFN$_\gamma$ dependent chemokine CXCL11, in dermal fibroblasts, without altering their viability.
and proliferation. Moreover, our study confirms that IFNγ and TNFα stimulated in a different way, the secretion of CXCL8, or CXCL11, chemokines as expected (18). Interestingly, V₂O₅ can synergize with IFNγ and TNFα, furtherly increasing CXCL11 secretion. In addition, V₂O₅ combined with TNFα, elicited a synergistic influence on CXCL8 chemokine production, abolishing the inhibitory effect of IFNγ.

These results, on the whole, agreed with the view that V₂O₅ is able to induce and perpetuate an inflammatory disorder in the dermal tissue inducing inflammatory chemokines secretion (13).

Our findings regarding TNFα and IFNγ effect in fibroblasts are in line with the results of another study in a different type of cells. In fact, it has been recently investigated if CXCL8 and CXCL10 chemokines secretion by normal human thyrocytes, fibroblasts, colon epithelial cells, islet cells, and others (10,13,14,19-25). However, these cells are not able to produce the CXC chemokines in basal condition, but only when stimulated by cytokines, such as IFNγ and TNFα, that are released in a T-helper 1 (Th1) type inflammatory site, such as the thyroid at the beginning of Graves' disease, by Th1 activated lymphocytes. It has been suggested that this process can be involved in the initiation and the perpetuation of the inflammation in several autoimmune diseases (10,13,14,19-25), and considering our results it can be applied to the thyroid, too.

Our findings about vanadium stimulation of chemokines agree with those of other studies conducted in different cell types. V₂O₅ exposure is a cause of occupational bronchitis; a study evaluated gene expression profiles in human lung fibroblasts (in cultures) after V₂O₅ exposure with the aim to identify genes that could be implicated in the bronchial inflammation, repair, and fibrosis in the pathogenesis of bronchitis. Among

**Figure 7.** Treatment of dermal fibroblasts with V₂O₅ (100 nM) + IFNγ + TNFα stimulation significantly increased CXCL8 release (the Bonferroni-Dunn test was used for post-hoc comparison; *P<0.05 vs. IFN+TNF+V₂O₅; **P<0.05 vs. TNF; °P<0.05 vs. IFN+TNF). V₂O₅, vanadium pentoxide; TNF, tumor necrosis factor; IFN, interferon.

**Figure 8.** CXCL11 release was dose-dependently stimulated by treating dermal fibroblasts with V₂O₅ (1, 10 and 100 nM). *P<0.05 vs. the control (the Bonferroni-Dunn test was used for post-hoc comparison). V₂O₅, vanadium pentoxide.

**Figure 9.** When dermal fibroblasts were treated with V₂O₅ (100 nM) + IFNγ CXCL11 release was significantly increased *P<0.05 vs. the control group. °P<0.05 vs. the TNF group. °P<0.05 vs. the V₂O₅ group (the Bonferroni-Dunn test was used for post-hoc comparison). V₂O₅, vanadium pentoxide; IFN, interferon.

**Figure 10.** CXCL11 release was significantly increased by treating dermal fibroblasts with V₂O₅ (100 nM) + IFNγ + TNFα *P<0.05 vs. the control group. °P<0.05 vs. the IFN+TNF group (the Bonferroni-Dunn test was used for post-hoc comparison). V₂O₅, vanadium pentoxide; TNF, tumor necrosis factor; IFN, interferon.
the 10 genes overexpressed by V$_2$O$_5$, also CXCL8, CXCL9 and CXCL10 were induced (26).

Another study reports that fibroblasts have a role in the innate immune response to vanadium-induced oxidative stress through the synthesis of IFNβ and the activation of STAT-1 that cause an increase of CXCL10 levels (27).

Interestingly vanadium can increase chemokine secretion in a dose range, from 1 to 100 nM. It could be observed that normal blood levels of vanadium are ranging from 0.45 to 18.4 nM, and that 100 nM is a dose that might mimick an abnormally high exposure (28). So we could hypothesize that the induction of an inflammatory reaction into the dermal tissue could predispose to the appearance of skin rashes, or atopic dermatitis.

In conclusion our study shows that V$_2$O$_5$ can induce CXCL8, and CXCL11 chemokines secretion into the dermal fibroblasts. Interestingly, V$_2$O$_5$ synergistically increased the effect of the IFNγ on CXCL11 secretion. Moreover, V$_2$O$_5$ synergistically increased the effect of the TNFα on CXCL8 secretion, abolishing the inhibitory effect of IFNγ. Overall CXCL8, and CXCL11 chemokines induction by V$_2$O$_5$ could lead to the appearance and perpetuation of an inflammatory reaction into the dermal tissue. Further studies are needed to evaluate dermal integrity, and manifestations in subjects occupationally exposed, or 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, SB, AA and SMF made substantial contributions to the conception and design of the study and to the acquisition of data. All authors analyzed the data. PF, SB, AA and SMF drafted the manuscript. AA revised the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the study are appropriately investigated and resolved. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all study participants and the study was approved by the University of Pisa Ethics Committee.

Consent for publication

Written informed consent was obtained from all participants for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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