

Adsorption of Lipoproteins onto Mineral Dust Surfaces: A Possible Factor in the Pathogenesis of Particle-induced Pulmonary Fibrosis?

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We compare the adsorption behavior of high density lipoproteins (HDL) and low density lipoproteins (LDL) on “fibrogenic” and “nonfibrogenic” mineral dusts. The adsorption tests with bovine lipoprotein concentrate and human serum produced the following results: 1) All seven examined fibrogenic dusts (SiO₂ DQ12, SiO₂ F600, silica, graphite, TiC, kaolin, talc) adsorbed significantly more high density lipoproteins (HDL), than the five examined nonfibrogenic (inert) dusts (TiO₂, SnO₂, Al₂O₃, Fe₂O₃, Fe₃O₄). This different behavior was particularly conspicuous in the presence of competing adsorbates (serum proteins). 2) In contrast, the adsorption of LDL did not correlate with the fibrogenicity of the mineral dusts. 3) The known silicosis-protective substance polyvinylpyridine-*N*-oxide inhibits the HDL adsorption of α -quartz. These results indicate that the adsorption of HDL could have a causal relationship with the triggering of a fibrotic reaction. The adsorption on the surface of fibrogenic dust particles provides an exceptional opportunity for the intake of HDL by macrophages. During the phagocytosis of the inhaled dust particles, the HDL adsorbed on the surface of the particles could be taken up by macrophages regardless of the receptor. There the HDL particles and/or compounds associated with them, such as lecithin-cholesterol-acyltransferase, could stimulate the macrophages to release fibrogenic mediators by some yet unknown mechanism.

Key words: Fibrosis, Mineral Dusts, High Density Lipoproteins

Introduction

Fibrosis is a complex tissue response whose predominant characteristic is the excessive deposition of extracellular collagens (Trojanowska *et al.*, 1998). It can be initiated in a great variety of ways, such as by inhaled dust particles, *e.g.* quartz (Allison, 1996), or by implanted foreign bodies, *e.g.* plates or foils made of nondegradable materials, such as plastic or precious metals (Bischoff and Bryson, 1964; Brand, 1982; Bonfield *et al.*, 1992). Tissue fibrosis demonstrates remarkable parallels with the normal wound healing process, but the resolution of scarring is impaired (Mutsaers *et al.*, 1997). While the importance of macrophages as the initial target cell of fibrogenic dust particles or fibrogenic implants is undisputed, the detailed mechanism by which macrophages are specifically

stimulated and mediate further inflammatory responses resulting in fibrosis is not clear (Kane, 1996). We justifiably assume that the surfaces of all fibrogenic particles have a specific property which is lacking in the nonfibrogenic (inert) particles or is at least significantly less effective. After noticing by chance that quartz (SiO₂) conspicuously adsorbs the lipoproteins of a serum, we attempted to clarify whether the preferred adsorption of lipoproteins represents a common property of the *fibrogenic* mineral dusts, possibly distinguishing them from the *nonfibrogenic (inert)* mineral dusts.

Materials and Methods

Equipment and chemicals

Infrared Fourier Transform Spectrometer 1720 (Perkin-Elmer); Photometer, ce 1010 (Cecil Instruments, Cambridge); nonadecanoic acid (Sigma, N-5252); *n*-hexadecane (Sigma, H-0255); lipoproteins from bovine plasma (Sigma, L-3626); for photometric determination of cholesterol: CHOD-PAP

Abbreviations: HDL, high density lipoprotein; HDL-C, HDL-cholesterol; LDL, low density lipoprotein; LDL-C, LDL-cholesterol; NDA, nonadecanoic acid; LCAT, lecithin-cholesterol-acyltransferase; PVPNO, polyvinylpyridine-*N*-oxide; apoA-I, apolipoprotein A-I.

method (Ecoline 25, Merck, 14830); precipitating reagents for HDL and LDL determination (Merck, 14210 and 14992); calibrator (Merck, 19720); polyvinylpyridine-*N*-oxide (PVPNO) (Prof. F. Pott, University of Düsseldorf, Germany); two quantities of quartz sand with the surface areas $S_1 = 0.299 \text{ m}^2/\text{g}$ and $S_2 = 0.406 \text{ m}^2/\text{g}$ [calibration substances for Blaine devices of the Amtliche Materialprüfanstalt für Steine und Erden (German testing agency for minerals and earth) Clausthal-Zellerfeld, Germany]. “Fibrogenic” mineral dusts: α -quartz DQ 12 (German quartz standard, Prof. F. Pott, University of Düsseldorf); α -quartz F 600 (French quartz standard, Prof. F. Pott, University of Düsseldorf); silica (Sigma, S-5631), graphite (C) (Merck, 4206); titanium carbide (TiC) (Goodfellow, Ti 546010/1); kaolin ($\text{Al}_4[(\text{OH})_8/\text{Si}_4\text{O}_{10}]$) (Sigma, K-7375); talc ($\text{Mg}_3[(\text{OH})_2/\text{Si}_4\text{O}_{10}]$) (Sigma, T-2015). “Nonfibrogenic” (“inert”) mineral dusts: anatase (TiO_2) (Sigma, T-8141); tin(IV) oxide (SnO_2) (Merck, 7818); alumina (α - Al_2O_3) (Sigma, A-2039); hematite (α - Fe_2O_3) (Merck, 3924); magnetite (Fe_3O_4) (Sigma-Aldrich, 31.006–9).

Surface area determination

We determined the surface area using an infrared spectrometrically monitored adsorption of nonadecanoic acid (NDA) from a solution in *n*-hexadecane. A weighed quantity (m_P) of the mineral dust (e.g. 500 mg) was shaken with 10 ml of solution A (NDA in *n*-hexadecane, $\gamma = 1.50 \text{ mg/ml}$) for 30 min at room temperature. The suspension was then centrifuged for 5 min at $1000 \times g$ and the clear supernatant was provided as solution C for the extinction measurement. The extinction measurements were performed against *n*-hexadecane in a NaCl-cuvette (0.1 mm) in the maximum of the CO-oscillation at 1714 cm^{-1} . For the mass m_X the adsorbed acid is:

$$m_X = 5.00 (E_A - E_C)/(E_A - E_B) [\text{mg}]. \quad (1)$$

Here E_A is the extinction of solution A (NDA in *n*-hexadecane, $\gamma = 1.50 \text{ mg/ml}$), E_B is the extinction of solution B (NDA in *n*-hexadecane, $\gamma = 1.00 \text{ mg/ml}$), E_C is the extinction of solution C (supernatant made of the experiment, see above). When m_X exceeded the value of 3 mg, it was repeated with a smaller sample quantity to increase the precision of the determination. With knowledge of the packing density of the adsorbed NDA in a monolayer (2.58 mg/m^2 , see Results), the sur-

face area S_{NDA} of the sample could be calculated according to the following equation:

$$S_{\text{NDA}} = 1000 m_X / 2.58 m_P [\text{m}^2/\text{g}]. \quad (2)$$

Here m_P is the mass (mg) of the employed dust sample and m_X the mass (mg) of the adsorbed acid calculated according to (1).

Adsorption of the lipoproteins

The adsorption experiments were carried out a) with commercial bovine lipoprotein concentrate that was predominantly made of HDL stock (HDL-C 90.8%; LDL-C 8.1%) according to manufacturer specifications and b) with human serum. The serum employed was made fresh daily from the extracted blood of a 68-year-old male and had the following composition during the test series: HDL-C, $(0.59 \pm 0.05) \text{ mg/ml}$; LDL-C, $(1.02 \pm 0.08) \text{ mg/ml}$.

Mineral dust with the mass that corresponds to the surface area of 1 m^2 was mixed with 3 ml of lipoprotein solution or serum and shaken for 1 h. Subsequently the suspension was centrifuged for 20 min at $1000 \times g$. The total cholesterol in the supernatant, as well as the cholesterol of the high density lipoproteins (HDL-C) and the low density lipoproteins (LDL-C) could be photometrically determined. The difference to initial concentration revealed the quantity of the adsorbed lipoproteins on the 1 m^2 -surface.

Results

Surface area of the mineral dusts

The quartz sands we employed for calibration adsorbed the NDA made of an almost saturated solution with a maximum of $(2.58 \pm 0.12) \text{ mg/m}^2$ (Fig. 1). The space requirements of a NDA molecule on the surface of these sand particles is $(0.192 \pm 0.009) \text{ nm}^2$. This value corresponds to the space requirements of long-chained fatty acids of a monolayer that numerous authors have specified as $\approx 0.2 \text{ nm}^2$ (Davis *et al.*, 1940; Kuhn, 1967; Small, 1984).

The quartz sands we employed for calibration are therefore as good as pore-free and the film of the adsorbed NDA is unambiguously monomolecular. Assuming that all the other dusts we examined could also adsorb NDA in a monomolecular layer with 2.58 mg/m^2 , the surface areas S_{NDA} of these dusts were determined (Table I). The values lie between $0.3 \text{ m}^2/\text{g}$ and $7.7 \text{ m}^2/\text{g}$. The large differ-

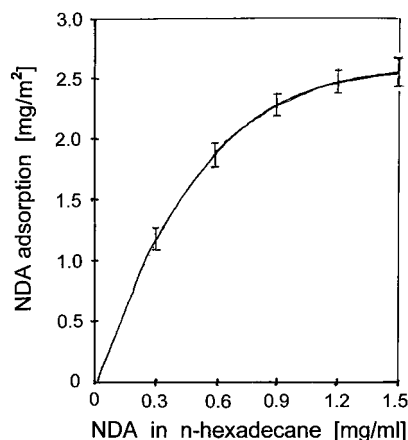


Fig. 1. Adsorption isotherm of nonadecanoic acid (NDA) in *n*-hexadecane on quartz sand at $(22 \pm 1)^\circ\text{C}$. The packing density of NDA in the monolayer is $(2.58 \pm 0.12) \text{ mg/m}^2$. (Data are means $\pm$ S. D. of three experiments.)

ences distinctly show how important a knowledge of the specific surface is for a meaningful comparison of the adsorption characteristics of dusts.

Adsorption of lipoproteins

In our experiments with a bovine lipoprotein concentrate, we refrained from separately determining the adsorption of HDL-C and LDL-C, and determined only the adsorption of total cholesterol. The result of the adsorption experiments with a series of fibrogenic and nonfibrogenic mineral dusts is presented in Fig. 2. The statistical evaluation shows that the lipoproteins – primarily HDL – contained in the concentrate are signifi-

Table I. Surface areas (S_{NDA}) determined with the method of NDA adsorption.

Mineral dust	S_{NDA} [m^2/g]
α -Quartz, DQ 12 (α - SiO_2)	3.13 ± 0.45
α -Quartz, F 600 (α - SiO_2)	1.52 ± 0.19
Silica (Sigma) (α - SiO_2)	2.38 ± 0.22
Graphite (C)	0.63 ± 0.11
Titanium carbide (TiC)	0.30 ± 0.02
Kaolin ($\text{Al}_4[(\text{OH})_8/\text{Si}_4\text{O}_{10}]$)	7.69 ± 0.97
Talc ($\text{Mg}_3[(\text{OH})_2/\text{Si}_4\text{O}_{10}]$)	1.92 ± 0.17
Anatase (TiO_2)	7.14 ± 0.60
Tin(IV) oxide (SnO_2)	7.14 ± 0.87
Alumina (α - Al_2O_3)	0.36 ± 0.07
Hematite (α - Fe_2O_3)	2.22 ± 0.22
Magnetite (Fe_3O_4)	1.04 ± 0.05

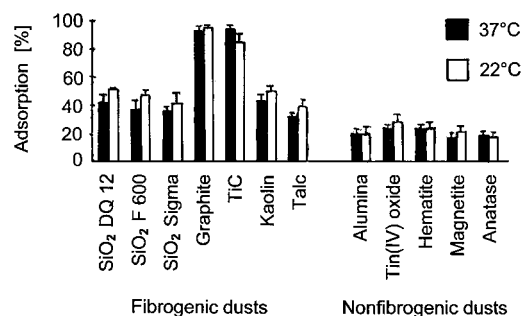


Fig. 2. Lipoprotein adsorption (measured as total cholesterol) from the solution of bovine lipoprotein concentrate ($\geq 90\%$ HDL) on 1 m^2 surface of fibrogenic and non-fibrogenic mineral dusts at 22°C and 37°C . The cholesterol content of this solution was $(1.90 \pm 0.11) \text{ mg/ml}$. (Data are means $\pm$ S. D. of three experiments.)

cantly more adsorbed on the fibrogenic mineral dusts than on the nonfibrogenic dusts (Mann-Whitney U-Test, $p = 0.003$).

The especially strong adsorption of graphite (C) and titanium carbide (TiC) in comparison to quartz (SiO_2) is striking in this case. Naturally, this behavior is also distinctly presented in the comparison of the adsorption isotherms (Fig. 3). The course of the isotherms is surprising because in this case all isotherms travers a maximum. Such a phenomenon has already been observed for a few

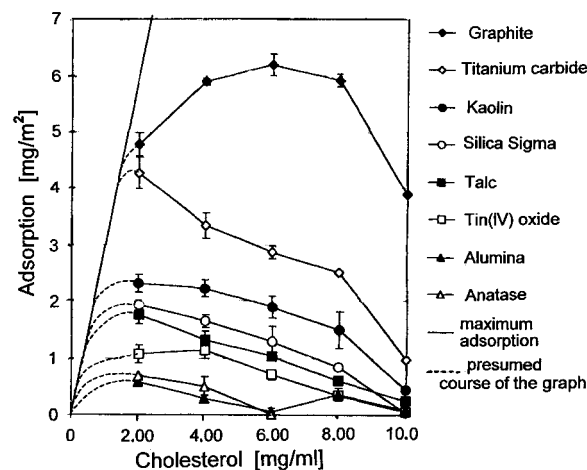


Fig. 3. Adsorption isotherms of lipoproteins (measured as total cholesterol) from a bovine lipoprotein concentrate ($> 90\%$ HDL-C) at $(22 \pm 1)^\circ\text{C}$. The solution was diluted with 0.9% Nall. (Data are means $\pm$ S. D. of three experiments.)

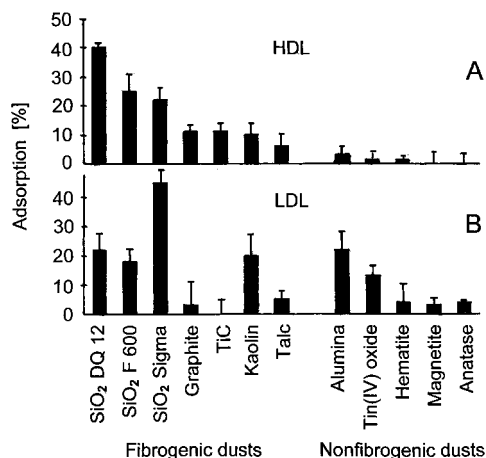


Fig. 4. A) HDL-adsorption (measured as HDL-C) and B) LDL-adsorption (measured as LDL-C) from human serum with HDL-C = (0.59 ± 0.05) mg/ml and LDL-C = (1.02 ± 0.08) mg/ml on 1 m^2 of fibrogenic and nonfibrogenic mineral dusts ($T = 37^\circ \text{C}$). (Data are means $\pm$ S.D. of three experiments.)

other substances and described as the “Vroman effect” (Poot *et al.*, 1990). In our case we surmise that the HDL particles aggregate in solutions of high concentration and then are no longer completely available as a specific adsorbate. Such high HDL concentrations as in this case of maximum isotherms are however not physiological, and therefore this “Vroman effect” should not concern us here.

The results of the adsorption experiments with human serum are presented in Fig. 4. The behavior of HDL and LDL varies considerably. HDL is primarily adsorbed by the fibrogenic dusts, while the nonfibrogenic (inert) dusts only slightly adsorb HDL. Merely 0–3% of the HDL contained in the serum are adsorbed on the surface of the inert dusts.

In comparison, the fibrogenic dusts adsorb up to 40% of the HDL. It is especially notable that the quartz, which possesses the highest fibrogenic capability among the examined substances, also has the strongest HDL adsorption by far. In comparison, the capability for adsorption of LDL is entirely independent of fibrogenic capability of the dusts. The strongest LDL-adsorption is shown by silica (Sigma) with 45%. In comparison, the likewise fibrogenic titanium carbide (TiC) adsorbed no LDL at all under the same conditions. The visual comparison of both diagrams in Fig. 4

shows very distinctly that the examined lipoproteins alone correlate the HDL adsorption with the fibrogenic capability of the dust.

We were interested in the question whether the antislipogenic polyvinylpyridine-*N*-oxide (PVPNO) (Schlippkötter *et al.*, 1963) was capable of arresting the adsorption of the lipoproteins on α -quartz. These experiments were distinguished from the previous ones only by the addition of 100 mg PVPNO to 10 ml serum. All other test conditions remained unchanged. The results corresponded to our expectations insofar as the adsorption of HDL on α -quartz in fact was drastically inhibited (Fig. 5). For standard quartz DQ 12 and F 600, the inhibition under these conditions was more than 90%, for silica from Sigma Company still 49%. The LDL adsorption was very unevenly influenced. The inhibition of the LDL adsorption on DQ 12 was still the strongest with 65%, on F 600, however, amounted to only 11%. On silica from the Sigma Company, the LDL adsorption was however even reinforced by 26%. The reasons for this enhancement are still unknown.

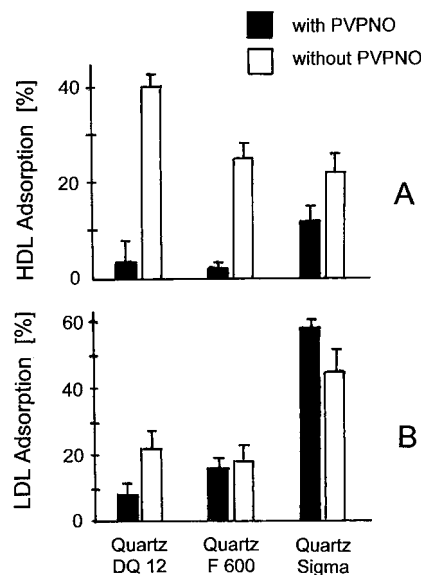


Fig. 5. A) HDL-adsorption (measured as HDL-C) and B) LDL adsorption (measured as LDL-C) from human serum [HDL-C = (0.59 ± 0.05) mg/ml and LDL-C = (1.02 ± 0.08) mg/ml] with and without addition of PVPNO (10 mg/ml) at three different α -quartz. (Data are means $\pm$ S.D. of three experiments.)

Discussion

HDL is more strongly adsorbed by fibrogenic than by nonfibrogenic dusts (Fig. 2) and this is reinforced by the presence of competing adsorbates (Fig. 4). This observation confirms earlier results of McFee and Tye (1965), who determined with the immunoelectrophoresis method that the "fast lipoprotein" – presumably HDL – was selectively adsorbed by α -quartz and cristobalite, but it was not adsorbed by the "inert" dusts ferric oxide and emery. The adsorption of the lipoproteins of the serum is influenced by the presence of competitors. Serum contains competing adsorbates, *i.e.* components (such as proteins), which likewise more or less strongly adsorb on the surface of the dust particles and can displace lipoproteins. It is therefore possible that a mineral dust that adsorbs large quantities of HDL (> 90%) made of one relatively "clean" HDL concentrate (*e.g.* graphite, titanium carbide; see Fig. 2 or 3), binds considerably less HDL, *i.e.* only 12% from the serum, when the surface of other adsorbates is engaged with greater affinity and/or concentration (see Fig. 4). Inhaled particles, however, first of all do not come in contact with serum, but rather with a pulmonary surfactant of an aqueous mixture of lipids (mostly phospholipids), carbohydrates and proteins. Specific components of pulmonary surfactants, especially dipalmitoyl phosphatidyl choline (DPPC) are, for example, adsorbed by the fibrogenic dusts α -quartz and kaolin in the *in vitro* experiment, whereby the strong membranolytic activity of these dusts is eliminated. As competing adsorbates, these components are certainly in the position to influence the adsorption of lipoproteins. It is interesting that DPPC is more tightly bound on the modestly fibrogenic kaolin than the strongly fibrogenic α -quartz (Wallace *et al.*, 1992).

It appears that HDL and LDL require various characteristics of the particle surface for adsorption. HDL apparently competes with the hydrogen acceptor PVPNO for the acidic silanol groups ($\equiv \text{SiOH}$) on the surface of the quartzes. Also hydrophobic surfaces (graphite, TiC) appear to be especially suitable for the adsorption of HDL (see Figs. 2 and 3). In comparison, LDL preferred the amphoteric or basic surfaces [alumina ($\alpha\text{-Al}_2\text{O}_3$), tin(IV) oxide (SnO_2), kaolin ($\text{Al}_4[(\text{OH})_8/\text{Si}_4\text{O}_{10}]$), see Fig. 4]. Both characteristics of the HDL-binding surfaces, namely a) a hydrogen-bond forming group (*e.g.* $-\text{OH}$) and b) a hydrophobic region, are present in cholesterol and are required for the

binding to the amphipatic helix of apoprotein A-I (apoA-I), the principal constituent of the HDL (Klimov *et al.*, 1992). From this we draw the conclusion that apoA-I could also be responsible for the adsorption of HDL at fibrogenic surfaces.

The absorption of oxidatively-modified LDL by macrophages is a factor in the pathogenesis of arteriosclerosis. It has not been clarified whether macrophages are in the position of also taking up HDL particles. The scavenger-receptor (SR) responsible for the internalization of LDL in any case does not bind native HDL (Parthasarathy *et al.*, 1990; La Ville *et al.*, 1994). For HDL the adsorption at the surface of fibrogenic particles perhaps is the only possibility of being taken up by macrophages. The dust particles loaded with HDL could then have a carrier function, by bringing HDL into the macrophages in the course of their phagocytosis. Possibly this is how internalized HDL stimulate the macrophages to release fibrogenic mediators. The mechanism of such stimulation is however still entirely puzzling. Is it perhaps an oxidatively modified HDL or an enzyme associated with HDL (*e.g.* lecithin-cholesterol-acyltransferase, LCAT) that causes the stimulation of the macrophages?

Nonfibrogenic (inert) dusts likewise adsorb HDL, if only in such small quantities that they apparently do not suffice for a fibrotic reaction. If these dusts are phagocytosed in large quantities by the macrophages, this leads to an "overloading" of the macrophages (Morrow, 1994). Under these circumstances the large surface of these dust masses can transport HDL in a quantity that must be sufficient for stimulating the macrophages. This hypothesis would be a plausible explanation for the fact that upon "overloading" of the macrophages, even "nonfibrogenic" (inert) dusts can induce a fibrosis.

The hypothesis supported by the findings of the present work, that the adsorption of HDL presents a factor in the development of a particle-induced fibrosis, will possibly enlarge on fibrogenic solids in general and is not alone limited to mineral dusts. The circumstance that HDL was determined to be the preferred adsorbate on diverse synthetic materials (Bagdade and Albers, 1977; Noishiki, 1982; Breemhaar *et al.*, 1984; Poot *et al.*, 1990; Van Damme *et al.*, 1991; Feng *et al.*, 1996), points in this direction. During the unsuccessful experiments to phagocyte an implant, the assembled macrophages on the implant surface are pos-

sibly capable – perhaps with the help of their own opsonins – to take up the adsorbed HDL particles. These events may lead to the stimulation of the macrophages with following fibrous tissue encapsulation of the implanted nondegradable material. The phagocytosis of the HDL is possibly aggravated on rough implant surfaces, so that in these cases the beginning of a fibrosis is delayed. This concept requires further experimental investigations.

Driscoll *et al.* (1990) have determined that the presence or absence of serum is an important factor influencing macrophage responses. This must also be the case when HDL – a component of the serum – should actually participate at the origination of the process of fibrosis. In one serum-free *in vitro* system, the experiment of a stimulation of human alveolar macrophages for the release of fibrogenic mediators even with the strongly fibrogenic and cytotoxic α -quartz was not successful. In

comparison, in the presence of serum, α -quartz was only minimally cytotoxic but stimulated the release of fibrogenic cytokines like IL-1 and TNF- α (Jabbour *et al.*, 1996). In numerous *in vitro* assays (Miller and Anderson, 1989; Bonfield *et al.*, 1992; Cardona *et al.*, 1992) a serum was added to the culture medium for the stimulation of the macrophages with biomedical polymers. Positive test results with a macrophages culture without serum or HDL are hitherto unknown to us.

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