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The production of anti-inflammatory cytokines in whole blood by physico-chemical induction

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Abstract. *Objective and design:* Cytokines such as interleukin-1 (IL-1) play an important role in degenerative musculo-skeletal diseases, including osteoarthritis, and a multitude of inflammatory disorders. Agents that inhibit the action of such cytokines have a high therapeutic potential in such diseases. Here we describe a new method for enhancing the production of the interleukin-l receptor antagonist (IL-1Ra) and other anti-inflammatory cytokines in whole blood.
Material and methods: Human venous blood was incubated in the presence of $CrSO_4$-treated glass beads. Serum was recovered and the concentrations of IL-1Ra and other relevant cytokines were measured by ELISA.
Results: The interaction of the glass bead surface with cells in whole blood increased production of IL-1Ra and anti-inflammatory cytokines. Removal of the beads and centrifugation generated a serum preparation enriched in anti-inflammatory cytokines. This preparation is of therapeutic value in treating various inflammatory and degenerative disorders.
Conclusions: The increased de novo production of anti-inflammatory cytokines by a direct physico-chemical induction of whole blood in the Orthokin system is feasible and offers an alternative, novel approach to treating mild to moderate OA and other orthopaedic conditions such as degenerative spine diseases.

Key words: Cytokine – Degenerative spine disease – IL-1Ra – Osteoarthritis – Orthokine

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Introduction

Interleukin-1 (IL-1) is a pivotal mediator of many inflammatory and degenerative diseases, including osteoarthritis (OA). The therapeutic use of IL-1 inhibitors in such diseases was proposed in the early eighties [1] and has formed the basis for the development of new biological treatment modalities [2–5]. Strategies for inhibiting the biological activities of IL-1 include use of the interleukin-1 receptor antagonist (IL-1Ra), soluble forms of IL-1 receptors, and type 1 cytokines such as interleukin-4 (IL-4), interleukin-10 (IL-10) and interleukin-13 (IL-13), that inhibit the synthesis of IL-1, increase the synthesis of IL-1Ra or do both. Here we describe a novel method for increasing the production of IL-1Ra and other anti-inflammatory molecules by whole blood. Arend and co-workers demonstrated the induction of IL-1Ra synthesis in purified monocytes after immunoglobulin G (IgG) stimulation [6–8]. As this technique is too laborious and expensive to be used therapeutically, we investigated alternative, more expeditious methods for stimulating IL-1Ra synthesis by blood cells. Here we report a method for the production of anti-inflammatory cytokines from whole blood samples in therapeutically relevant amounts by physico-chemical induction.

Materials and methods

Container and bead preparation

Blood was incubated in either microtiterplates (24 and 48 wells, Nunc, Denmark) or 60 ml syringes (Perfusor Syringes, Becton Dickinson, USA). The syringes contained 200 glass beads. Glass beads were 2.5 mm in diameter, had a surface area of 21 mm^2 and were of medical

grade. The beads were washed with sterile, double distilled water until the conductivity was less than 0.3 µS (Hanna Instruments, USA). The surface of the beads was modified by incubation in 50% v/v CrSO4 (Merck, Germany) for 5 min. The beads were then washed repeatedly until the pH was identical to that of the distilled water used for the rinsing and the conductivity was less than 0.3 µS (Hanna Instruments, USA). The microtiterplates or syringes were packed with beads and sterilised either by autoclaving or gamma-irradiation.

Blood culture techniques

In all experiments, containers (microtiterplates or 60 ml syringes) packed with beads were filled with freshly drawn human whole blood from healthy, male or female donors, between 20 and 50 years old, without anti-coagulants unless mentioned otherwise. Whole blood cultures were established under sterile, laminar flow conditions (Kendro, Germany). Incubation was carried out aseptically at 37 °C, 5% CO_2 (Kendro, Germany) for either 24 h or different intervals, as indicated in the legends. After incubation, serum was retrieved and centrifuged (3500 rpm, 10 min., Megafuge, Kendro, Germany). From the microtiterplates 200 µl and from the syringes 10 ml serum was retrieved, which corresponds to approximately 20% of the total original blood volume. The serum was stored at –20 °C.

Serum analysis

The cytokine levels in the serum were measured by ELISA. ELISA kits were purchased from R&D Systems (USA) and Biosource (USA) and employed according to the manufacturer's instructions. The presence of microbial contaminants (bacteria, fungi and mycoplasm) and serological parameters (human immunodeficiency virus (HIV) 1 & 2, hepatitis B virus (HBV), hepatitis C virus (HCV) and Syphilis) in serum produced in syringes were assessed by external clinical laboratories. Glucose and potassium (K+) values and serum protein composition were determined in a clinical laboratory.

Statistical analysis

Data were analysed using Microsoft Excel 2001 and SPSS 10 for Apple Macintosh. All results shown here are the means ± SE of two or more experiments. The statistical significance of differences was determined in paired sample t-tests.

Results

Induction of autologous anti-inflammatory cytokines

Exposure of blood to the treated glass beads elicited a vigorous, rapid increase in the synthesis of several anti-inflammatory cytokines, including IL-1Ra, IL-4 and IL-10. The concentration of IL-1Ra increased 140-fold during a 24 h incubation (Table 1). The concentrations of IL-4 and IL-10 were slightly induced by treatment with glass beads. Although incubation of the whole blood with the glass beads leads to production of anti-inflammatory cytokines, no increase in the levels of the pro-inflammatory cytokines Interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) was detected (Table 1). No significant alterations in protein composition occurred during the processing of the blood (Table 2). The strong consumption of glucose and weak haemolysis (as measured by a slight increase in potassium concentration, Table 2) indicate that the viability of the blood cells was maintained well during incubation.

Table 1. Cytokine induction in syringes containing glass beads.

Parameter	T = 0 h (pg/m1)	T = 0,5 h (pg/ml)	T = 24 h (pg/ml)	Increase (factor)
IL-4	8.1 ± 2.1	7.8 ± 2.3	17.2 ± 2.8	2.1*
IL-10	4.1 ± 1.1	4.3 ± 0.4	8.9 ± 1.2	2.2*
IL-13	187 ± 10.2	192 ± 13.0	189 ± 9.6	–
IL-1Ra	73 ± 4.8	778 ± 58	10254 ± 165	140*
IL-1β	< 3.9	< 3.9	< 3.9	–
TNF-α	< 15.6	< 15.6	< 15.6	–

Incubation of whole blood in the syringe elevates the levels of anti-inflammatory cytokines significantly (n = 44). The concentrations of pro-inflammatory cytokines TNF-α and IL-1β were below detection limit of the assay before and after incubation. The IL-1Ra concentration was strongly enhanced by this treatment. A significant increase in the concentrations of other anti-inflammatory cytokines (IL-4, IL-10) can also be observed (* = $p < 0.001$).

Table 2. Serum, glucose and potassium protein concentrations before and after incubation.

	T = 0 h	T = 24 h
Albumin (g/dl)	4.39 ± 0.18	4.44 ± 0.12
alpha-l-Globulin (g/dl)	0.18 ± 0.015	0.16 ± 0.018
alpha-2-Globulin (g/dl)	0.70 ± 0.041	0.68 ± 0.043
beta-Globulin (g/dl)	0.72 ± 0.043	0.71 ± 0.054
gamma-Globulin (g/dl)	0.84 ± 0.077	0.84 ± 0.065
Total protein (g/dl)	6.8	6.8
Glucose (mg/dl)	94 ± 6.0	35 ± 3.9*
K+ (mmol/l)	4.4 ± 0.094	8.6 ± 0.62*

Serum protein, glucose and K+ levels were measured in the same blood sample before and after 24 h incubation. During incubation of the syringe the concentration of the major serum protein components is not significantly altered, whereas the glucose concentration decreases and potassium concentration increases significantly (n = 9, * = $p < 0.001$). Glucose depletion to one third of the starting level indicates strong metabolic activity and indicates that cells survive and are intact during the process of 24 h incubation. The slight increase in K+ concentration indicates a moderate haemolysis that is typically seen after a 24 h incubation at 37 °C.

IL-1Ra is produced de novo by glass bead induction

The finding that increase of IL-1Ra expression starts within 30 min of blood withdrawal (Table 1) led to the question, whether IL-lRa is synthesized de novo. The observation that upon incubation the IL-lRa concentration steadily increases for 24 h (Fig. 1), suggests de novo synthesis. This was confirmed by the ability of cycloheximide to inhibit the accumulation of IL-1Ra (Fig. 3).

Discussion

The data presented here demonstrate the feasibility of producing autologous anti-inflammatory cytokines by a physico-chemical treatment of whole blood. By using whole blood instead of purified monocytes, and by stimulating cells with treated glass beads, the system lends itself readily to clinical application.

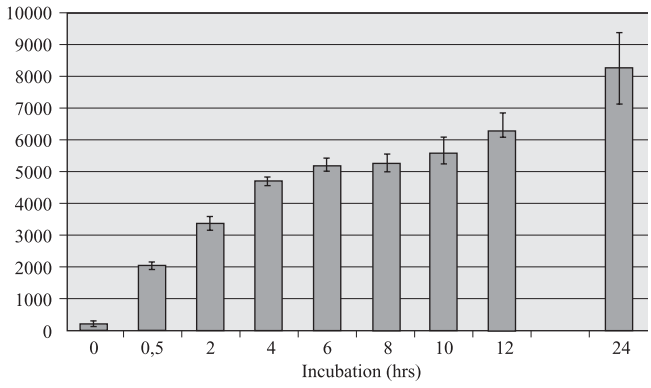

Fig. 1. Increase in IL-1Ra concentration as a function of incubation time. The IL-1Ra serum-level (pg/ml) ± SE (n = 3, triplicate measurements) increases within 30 min and reaches its maximum at 24 h. After 24 h the IL-1Ra concentration decreases (data not shown). As a container a 60 ml syringe was used.

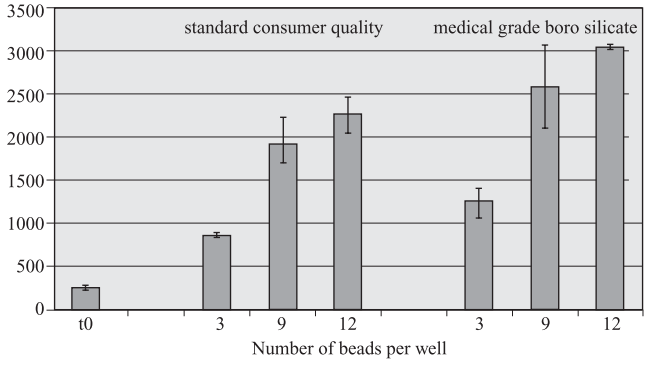

Fig. 2. IL-1Ra production as a function of bead number. The increase of IL-1Ra (pg/ml) in conditioned heparinized whole blood is dependent on the total number and composition (standard consumer quality versus medical grade boro silicate quality) of the glass beads (n = 3, triplicate measurements). Incubation was performed in a microtiterplate.

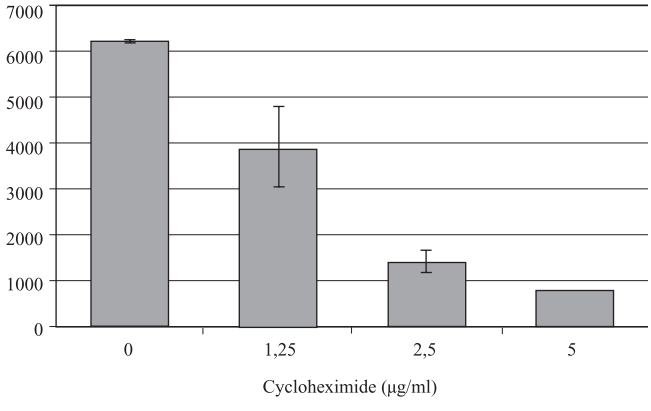

Fig. 3. IL-1Ra accumulation is inhibited by application of cycloheximide. Human whole blood was incubated as described in the presence of various concentrations of cycloheximide for 24 h in a microtiterplate. The accumulation of IL-1Ra (pg/ml) in whole blood is inhibited by cycloheximide (n = 3, triplicate measurements).

IL-1Ra induction in whole blood was as high as in cultivates of purified monocytes exposed to IgG [7] and occurred without the induction of IL-1β or TNF-α. Inhibition of IL-Ra production by application of cycloheximide (Fig. 3) indicates that the de novo cytokine synthesis is induced by the interaction between the glass surface and the blood cells. The magnitude of the increase in IL-1Ra concentration depended on the type of glass that was used (Fig. 2). Incubation with glass beads for 24 h does not lead to extensive cell death, as determined by a decrease in glucose concentration and the lack of IL-lβ and TNF-α production [9]. The potassium content increased only slightly, indicating that no severe haemolysis occurred. Additionally protein analysis shows that the major serum protein composition is not affected (Table 2).

Cytokine antagonists play an increasing role in the treatment of acute and chronic orthopaedic disorders such as rheumatoid arthritis (RA) and OA. Recombinant IL-lRa has been approved by the FDA as the drug Kineret for the treatment of RA. This molecule also inhibits the progression of experimental OA in dogs [10]. Moreover, intraarticular expression of the IL-1Ra cDNA inhibits the development of experimental OA in dogs [11], rabbits [10] and horses [12]. IL-1Ra also reduces pain and inflammatory reactions resulting from nerve root compression [4, 5, 10, 13–15].

A biologic therapeutic preparation known as 'Orthokine®' has been developed based on the principles described in this paper. Blood is drawn into a syringe containing treated glass beads. After incubation for 24 h at 37 °C, the blood is recovered and clarified by centrifugation. The autologous serum, now enriched for IL-1Ra, IL-4 and IL-10 is returned to the patient.

Since 1998, Orthokine® has been used clinically in orthopaedic patients suffering from OA, RA and spinal disorders. The results confirm the efficacy and high safety of the Orthokine® system [16]. One essential question is, whether the concentration of IL-1Ra in Orthokine® is sufficient for the treatment of human joints. The largest joint of the human body is the knee, having an average synovial fluid volume of 13.6 ml in OA [17]. The average IL-1 concentration measured in synovial fluids retrieved from an osteoarthritic knee joint is 2.5 pg/ml [18]. Conservatively assuming a concentration of IL-1Ra in Orthokine® of 3 ng/ml, a volume of 2 ml Orthokine® that is normally injected into knees with OA contains 6 ng of IL-1Ra. After one injection of Orthokine serum, the total volume of intraarticular fluid is increased to 15.6 ml. Thus the IL-1/IL1Ra ratio in the joint can be calculated as 1 : 170. A minimum IL-1/IL-1Ra ratio of 1 : 10 is required to inhibit IL-1 activity [19]. Thus, the Orthokine® injection has the capacity to inhibit strongly the biological actions of IL-1.

In many orthopaedic conditions, such as mild to moderate OA, there is a pressing need for new, non operative techniques to reduce pain, inhibit tissue degradation, and support tissue restoration. The current conservative armentarium is limited to palliation, cyclooxygenase inhibitors and viscosupplementation. Orthokine® is offering an alternative, novel approach to treating mild to moderate OA and other orthopaedic conditions such as degenerative spine diseases.

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