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BRIEF REPORT

Physico-Chemical Factors Influencing Autologous Conditioned Serum Purification

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Abstract

Autologous conditioned serum (ACS) is a recent biotherapy based on certain cytokines anti-inflammatory properties mainly used for the reduction of osteoarthritis (OA) symptoms. Here we investigated different physico-chemical factors influencing ACS purification and cytokine production. Human venous blood was incubated in the presence of different diameter beads (respectively 2.5, 3, 3.5, and 4 mm) or glass beads with different types of coating (polished or coated with $CrSO_4$). Sera were recovered, and the concentrations of pro-inflammatory and anti-inflammatory relevant cytokines were measured using Luminex® technology. Fresh whole blood incubated for 24 h highly increased production of interleukin (IL)-6 and IL-8 cytokines. At the same time, the concentrations of IL-1β, IL-1 receptor agonist (IL-1Ra), IL-10, and tumor necrosis factor (TNF)-α were slightly induced. The highest cytokine concentrations were obtained with the exposure of whole blood to 3-mm glass beads and 3.5-mm polished beads. The minimum IL-1β/IL-1Ra ratio obtained was 3.2 ± 1.3 after 24-h incubation without any beads. ACS has been shown to alleviate clinical symptoms of OA in clinical studies. This descriptive study demonstrated that different pro- and anti-inflammatory cytokines are present in ACS since no selective anti-inflammatory cytokines were produced based on the different protocols. Furthermore, we showed that $CrSO_4$-treated glass beads are not necessary and that the absence of beads combined with a 24-h incubation could also lead to an enriched serum.

Key words: autologous conditioned serum; Luminex®; osteoarthritis

Introduction

Interleukin (IL)-1β is a pivotal mediator of many inflammatory and regenerative diseases, including osteoarthritis (OA), rheumatoid arthritis (RA), and spinal disorders. Strategies for inhibiting the biological activities of IL-1β include use of the recombinant IL-1 receptor agonist (IL-1Ra, approved by the U.S. Food and Drug Administration as the drug Kineret® [Swedish Orphan Biovitrum, Stockholm, Sweden] for treatment of RA), soluble forms of IL-1 receptors, and anti-inflammatory cytokines such as IL-4, IL-10, and IL-13, which inhibit the synthesis of IL-1, increase the synthesis of IL-1Ra, or do both. Since 1998, a biologic therapeutic preparation known as autologous conditioned serum (ACS) or Orthokine (Orthokine, Düsseldorf, Germany) have been developed and used clinically in orthopedic patients suffering from OA, RA, and spinal disorders. ACS is prepared from peripheral whole blood. Briefly, blood is drawn into a syringe containing treated glass beads with $CrSO_4$ to initiate monocyte activation.[1] After incubation for 24 h at 37°C, the blood is recovered and clarified by centrifugation. The resulting autologous serum is now selectively enriched for anti-inflammatory cytokines IL-1Ra, IL-4, and IL-10 and returned to the patient.[2] ACS treatment consists of six repeated injections over a period of 21 days.

The intraarticular availability of adequate levels of IL-1Ra is important because IL-1β is considered to be active at low concentrations, and relatively high levels of IL-1Ra are required to inhibit the effects of IL-1β.[3] A minimum IL-1/IL-1Ra ratio of 1:10 is required to inhibit IL-1 activity.[4]

Here we investigated the extent to which different physico-chemical factors, including different bead diameters and different types of coating for glass beads, would influence ACS purification. We determined concentrations of anti-inflammatory (IL-8, IL-10, IL-1Ra) or pro-inflammatory (IL-1β, tumor necrosis factor [TNF]-α, IL-6) cytokines using Luminex® technology (Life Technology Corporation, Carlsbad, CA).

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Materials and Methods

Whole-blood source

Six healthy volunteer donors (three men and three women), between 25 and 45 years old (mean ± SD = 29.33 ± 7.81) with no relevant diseases were included in the study. For each donor, 60 mL of whole blood was collected in six 10-mL syringes without anticoagulants. Another tube (2.5 mL), which was coated with EDTA anticoagulant, was used to determine the initial formulation of the whole blood with an automatic cell counter (Advia 2120®, Siemens Diagnostic Solutions, Tarrytown, NY).

Bead preparation

Different diameter glass beads of medical grade and different types of coating were tested in this study: (1) 3 mm in diameter with a surface area of 28.27 mm^2 (VWR International, Radnor, PA); (2) 3.5 mm in diameter with a surface area of 38.48 mm^2 polished (VWR International); (3) 2.5 mm in diameter with a surface area of 21.00 mm^2 coated with $CrSO_4$ (Orthokine, Düsseldorf, Germany); and (4) 4 mm in diameter with a surface area of 50.27 mm^2 (VWR International). Ten-milliliter syringes were packed with 33 beads and sterilized through gamma-irradiation technology (Synergy Health, Les Arnavaux, Marseilles, France) according to the protocol described by Meijer et al.[2]

Preparation of conditioned serum

Syringes without beads or packed with the beads were filled with freshly drawn human blood. Whole-blood cultures were established under sterile, laminar conditions. Incubation was carried out aseptically at 37°C, 5% CO_2 for 24 h. After incubation, the sera were retrieved and centrifuged at $1000 \times g$ (Multifuge Heraus 3 S-R, ThermoScientific, Indianapolis, IN) for 10 min. Control syringes only containing whole blood (10 mL without glass beads without incubation) were immediately centrifuged. The sera were stored at −80°C until further use. All the experimental conditions tested are outlined in Table 1.

Cytokine analyses

The cytokine levels in the sera were detected and measured by Luminex technology. Luminex kits were used according to the manufacturer's instructions. All results were normalized to 50 beads and corrected with the background.

Statistical analysis

Data were analyzed using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL). All statistical tests were at the 5% level of significance.

Table 1. The Different Experimental Conditions

No.	*Experimental conditions*	*Incubation time (h)*
1	No bead	0
2	No bead	24
3	3-mm beads	24
4	3.5-mm polished beads	24
5	2.5-mm beads coated with $CrSO_4$	24
6	4-mm beads	24

The differences in mean cytokine concentration were analyzed by one-way analysis of variance (ANOVA) and Bonferroni *post hoc* testing is enabled to make pairwise comparisons of the preparations.

Results

Induction of autologous anti-inflammatory cytokines

Anti-inflammatory cytokines were undetectable in fresh sera. Exposure of blood to the different treated glass beads elicited a vigorous, rapid increase in the synthesis of IL-8 regardless of exposure conditions. Indeed, ANOVA showed no significant differences among the different conditions for IL-8, and IL-10 and IL-1Ra cytokines were only slightly induced. However, ANOVA and pairwise analysis using Bonferroni *post hoc* testing revealed significant differences for concentrations of these cytokines (respectively $p \leq 0.01$ and $p \leq 0.001$). IL-10 concentration (mean ± SD; pg/mL) was significantly higher after contact with 3.5-mm polished beads (594.1 ± 340.5) compared to the absence of beads (152.6 ± 186.1) and compared to the exposure to $CrSO_4$-coated 2.5-mm beads (167.0 ± 200.4). The IL-1Ra concentration was significantly higher after incubation with 3.5-mm polished beads (350.7 ± 136.7) compared to all the other conditions. All anti-inflammatory cytokine concentrations are reported in Table 2.

Induction of autologous pro-inflammatory cytokines

Fresh sera did not contain pro-inflammatory cytokines either. ANOVA and pairwise analysis using Bonferroni *post hoc* testing showed significant differences for IL-1β, TNF-α, and IL-6 concentrations (mean ± SD; pg/mL) according the different experimental conditions ($p \leq 0.0001$, $p \leq 0.01$, and $p \leq 0.001$, respectively). IL-1β concentration was significantly higher after exposure to 3.5-mm polished beads (1486.6 ± 454.1) compared to the absence of beads (319.4 ± 209.7), the exposure to 4-mm beads (601.9 ± 541.5), or 2.5-mm beads coated with $CrSO_4$ (252.5 ± 198.2). The TNF-α concentration was significantly higher after exposure to 3.5-mm polished beads (1180.8 ± 557.2) compared to the absence of beads (199.5 ± 325.7) and to the exposure to 2.5-mm beads coated with $CrSO_4$ (222.7 ± 393.4). The IL-6 concentration was significantly higher after exposure to 3.5-mm polished beads (11,771.7 ± 615.3) or 3-mm beads (11,681.7 ± 1010.7) compared to the absence of beads (5833.9 ± 3783.0) and compared to the exposure to 2.5-mm beads coated with $CrSO_4$ (5873.5 ± 3956.2). All pro-inflammatory cytokine concentrations are reported in Table 2.

IL-1β/IL-1Ra ratio

For each condition, we determined the IL-1β/IL-1Ra ratio and found values between 3.2 ± 1.3 and 5.2 ± 2.1 (Table 2). The lowest ratio was obtained under 24-h incubation without glass beads. ANOVA and pairwise analysis using Bonferroni *post hoc* testing showed no significant difference among the different conditions for the IL-1β/IL-1Ra ratio.

Discussion

Results showed that all the conditions tested have both raised anti- and pro-inflammatory cytokine concentrations

TABLE 2. ANTI-INFLAMMATORY AND PRO-INFLAMMATORY CYTOKINE CONCENTRATIONS (PG/ML) AND IL-1β/IL-1RA RATIO

	No incubation	*Incubation*					
Cytokines[a]	*No beads*	*No beads*	*3-mm beads*	*3.5-mm polished beads*	*4-mm beads*	*2.5-mm beads with $CrSO_4$*	*ANOVA*
Anti-inflammatory							
IL-8	2.5 ± 3.8	14,416.6 ± 1192.3	14,758.3 ± 602.0	14,525.0 ± 287.7	14,658.5 ± 335.3	14,300.0 ± 441.6	NS
IL-10	Undetectable	152.6 ± 186.1	426.8 ± 176.6	594.1 ± 340.5	221.8 ± 158.3	167.0 ± 200.4	≤ 0.01
IL-1Ra	Undetectable	98.3 ± 69.4	182.7 ± 77.6	350.7 ± 136.7	163.0 ± 112.4	71.5 ± 32.5	≤ 0.001
Pro-inflammatory							
IL-1β	Undetectable	319.4 ± 209.7	853.9 ± 318.1	1486.6 ± 454.1	601.9 ± 541.5	252.5 ± 198.2	≤ 0.0001
TNF-α	3.2 ± 1.0	199.5 ± 325.7	776.6 ± 496.9	1180.8 ± 557.2	569.3 ± 585.6	222.7 ± 393.4	≤ 0.01
IL-6	Undetectable	5833.9 ± 3783.0	11,681.7 ± 1010.7	11,771.7 ± 615.3	10,230.8 ± 3115.1	5873.5 ± 3956.2	≤ 0.001
IL-1β/IL-1Ra ratio (mean ± SD)	NA	3.2 ± 1.3	5.2 ± 2.1	4.7 ± 1.9	3.4 ± 2.2	3.3 ± 1.9	NS

[a]Values are means ± SD.
IL, interleukin; ANOVA, analysis of variance; TNF, tumor necrosis factor; SD, standard deviation; NS, not significant; NA, non-appropriate.

compared to the negative control, which corresponds to serum without exposure to any glass beads and without incubation. Regardless of exposure conditions, IL-8 and IL-6 cytokines showed the highest concentrations compared to IL-1β, TNF-α, IL-1Ra, and IL-10, which were more slightly induced. Exposure of blood to 3.5-mm polished beads and to 3-mm glass beads were the conditions providing the highest pro- and anti-inflammatory cytokine concentrations. However, these two conditions were the ones providing the highest IL-1β/IL-1Ra ratio. None of the conditions allowed obtaining a serum with an IL-1β/IL-1Ra ratio below 0.1, which has been described as the minimum to efficiently inhibit IL-1β through IL-1Ra. The lowest ratio was obtained under the condition in which blood was incubated for 24 h without any beads.

It is important to note that we obtained conflicting results with the initial publication on Orthokine[2] regarding the use of $CrSO_4$-treated glass beads. Indeed, Meijer et al.[2] described a resulting serum enriched in IL-1Ra and IL-10, with IL-1β and TNF-α below the detection baseline, whereas our results showed that exposure of blood to $CrSO_4$ glass beads increases all tested cytokines, not only the anti-inflammatory cytokines.

Our results were nearer to the ACS preparation of Rutgers and colleagues[5] in which both anti- and pro-inflammatory cytokines were increased following the use of Orthokine technology. However, in this same study, no adverse effect of ACS occurred on cartilage explant cultures, suggesting that anti-inflammatory and possibly chondroprotective ingredients predominate in ACS.[6]

Additionally, clinical data for ACS have shown positive results in several studies with regard to benefits in knee OA,[7] temporomandibular joint OA,[8] hip OA,[9] coxarthrosis,[10] and the decrease of bone tunnel widening after anterior cruciate ligament reconstructive surgery.[11]

Finally, this descriptive study includes a number of weaknesses including the coexistence of several variable factors, which makes it difficult to draw any conclusion about the more efficient way to produce ACS. Future research should focus on the complex interactions (synergism or antagonism) of cytokines that increase in ACS to create a solid basis for biomolecular treatment.

Notwithstanding these limitations, and because ACS has been shown to alleviate clinical symptoms of OA, the present study suggests that factors other than IL-1Ra alone may be involved in its clinical efficacy. Furthermore, our study showed that the use of $CrSO_4$-treated glass beads to produce ACS is not necessary and that the absence of beads combined with a 24-h incubation is sufficient to produce an enriched serum.

Author Disclosure Statement

The authors state no conflicts of interest. The authors declare that the study has not been previously published and has not been submitted for publication elsewhere while under consideration.

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Abbreviations Used

ACS = autologous conditioned serum
ANOVA = analysis of variance
IL = interleukin
IL-1Ra = interleukin-1 receptor agonist
OA = osteoarthritis
RA = rheumatoid arthritis
TNF = tumor necrosis factor