In vitro generation of primary cultures of human hyalocytes

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Purpose: A growing number of studies on animal models have demonstrated that some ocular diseases are the result of the interaction between hyalocytes and the ocular inflammatory setting. Endogenous and exogenous substances might alter the structure and behavior of hyalocytes that can contribute to the pathogenesis of some ocular diseases. Obtaining primary cultures of human hyalocytes could help understand the role of these cells in response to different treatments.
Methods: Hyalocytes were isolated from eyes of 54 patient volunteers subjected to vitrectomy for different clinical reasons. By testing different matrices and growth media, we reproducibly generated primary cultures of hyalocytes that we characterized morphologically and biologically, basally and upon treatment with several agents (basic fibroblast growth factor (bFGF), transforming growth factor beta 1 (TGF-β), platelet-derived growth factor subunit-BB (PDGF-BB), ascorbic acid, dexamethasone, and hydrogen peroxide).
Results: We were able to generate primary cultures from vitreous human samples, growing the cells on collagen-coated plates in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum; primary cells expressed the hyalocyte markers. Specific cytoskeletal modifications were observed upon treatment with bFGF, TGF-β, PDGF-BB, ascorbic acid, dexamethasone, and hydrogen peroxide. Only bFGF was able to promote cell growth and hyaluronic acid production.
Conclusions: We describe for the first time the generation and the characterization of primary cultures of human hyalocytes from living donors. Although human hyalocytes share some characteristics with animal hyalocytes, human hyalocytes have their own features typical of the species, confirming how important human experimental models are for investigating human pathologies and their treatments.

The vitreous body, the main component of the posterior eye, is a gel-like structure mainly consisting of collagen type II and hyaluronic acid (HA), with water content of about 98%. The ultrastructural organization of the vitreous body is characterized by a matrix of collagen fibers, separated from each other by HA molecules [1-3]. Within the vitreous body is also present, albeit minimally, a cellular component consisting of astrocytes, glial cells, and hyalocytes [4], mainly arranged on the cortex of the vitreous [4-6]. Although the function inside the vitreous body of the latter types of cells is not entirely known, data obtained in studies conducted on animal hyalocytes have shown that these cells could play an important role in many vitreous-retinal diseases, particularly in inflammatory and vitreous-retinal proliferative diseases [7,8]. Moreover, some studies have demonstrated that hyalocytes belong to the monocyte/macrophage lineage as they express the leukocyte common antigen (CD45), the lymphocyte function-associated antigen (CD11a), the Fc-gamma receptor 1 (CD64), and the hemoglobin (Hb) scavenger receptor (CD163), whereas they do not react with

antibodies against CD68, an antigen that is expressed by all tissue macrophages, or CD11b and CD14, antigens that are variably expressed by the monocyte/macrophage lineage [9-12]. It has been hypothesized that hyalocytes modify their biologic behavior in response to environmental changes: An inflammatory environment stimulates hyalocytes to gain a contractile function, which plays a fundamental role in the pathogenesis of macular pucker and macular hole [8,13-16].

Although in several studies animal hyalocytes have been isolated and characterized in terms of proliferation and in vitro behavior, to our knowledge, vital primary cultures from human hyalocytes have never been described. Some researchers, between 1980 and 2015, were able to isolate human hyalocytes to study their morphology, their immunohistochemical markers, and their role in proliferative vitreous-retinal diseases [9,13,14,17]. Nevertheless, these researchers did not succeed in obtaining vital cell cultures, and thus, they could not investigate modifications in the behavior of hyalocytes in the presence of environmental changes [9,13,14,17].

The main aim of this study was to provide a standardized and reproducible method for isolating and growing human hyalocytes from living donors, to characterize the cellular morphology, and to investigate the proliferation rate and HA production after different drug treatments. This study aims at

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better understanding the functions of human hyalocytes and their role in several vitreous-retinal diseases and highlighting the differences between human and animal hyalocytes.

METHODS

Reagents: Unless otherwise specified, reagents were purchased from Sigma-Aldrich (Milan, Italy), whereas plasticware was from Falcon (Becton Dickinson, Franklin Lakes, NJ).

Subjects: The study was performed in accordance with the Declaration of Helsinki for medical research involving human subjects in the period from February 2017 to June 2020 and was authorized by the local Ethical Committee (ref. number: 0077554). Signed, written informed consent was obtained from all patients accepting their enrolment in this study. All patients underwent a baseline ophthalmological examination before vitrectomy, including measurements of best-corrected visual acuity (BCVA), Goldmann applanation tonometry, and fundus examination. Fifty-four otherwise healthy patients with a history of vitreous-retinal diseases were randomly chosen among patients who underwent a 25 gauge or 27 gauge pars plan vitrectomy from the central vitreous cavity, for vitreoretinal disorders. The patients' characteristics are listed in Table 1. The following clinical characteristics were recorded, and patients presenting one or more of the criteria were excluded from the study: presence of vitreous hemorrhages or hemovitreous, assumption of antioxidant integrators, impossibility of classifying vitreous degeneration or any of the other relevant parameters, infections, malignant neoplasia, or renal or hepatic failure. Patients presenting non-physiologic levels of glycemia and fibrinogen were also excluded.

Vitrectomy and preparation of vitreous cell primary culture: From each patient, vitreous samples (500–800 µl) were collected from the central vitreous cavity, at the beginning of the 25 or 27 gauge pars plana mini-invasive vitrectomy with non-contact technique (BIOM). Samples were stored in a 15 ml tube containing Iscove's modified Dulbecco's medium (IMDM; Waltham, MA) supplemented with 10% fetal bovine serum (FBS), 1% glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. To increase the rate of hyalocyte isolation from the vitreous body, vitreous samples were incubated for 1 h at 37 ° C in a shaking incubator with 1 mg/ml collagenase (Sigma, Milan, Italy) diluted in IMDM. After the digestion, the vitreous bodies were centrifuged at 300 ×g for 5 min. The obtained pellet was resuspended in IMDM, and collagenase-treated and -free cells were seeded for primary culture in a six-well plastic culture plate coated with collagen I (Cellware, Corning, BioCoat, VWR, Radnor,

PA), and cultured at 37 ° C in an incubator in the presence of 5% CO_2 in the following different growth conditions: IMDM (+ 10% FBS) or stem cell medium [18] or F12 medium (+ 10% FBS). The medium was changed twice a week.

Cell proliferation assay: To evaluate cell viability, about 3,000 cells/well were seeded in triplicate in 96-well culture plates, in the presence of the different drugs. In each collagen-coated well, cells were seeded in 200 µl of 10% FBS IMDM containing the following agents: 10 ng/ml of basic fibroblast growth factor (bFGF), 5 ng/ml of transforming growth factor beta 1 (TGF-β), 10 ng/ml of platelet-derived growth factor subunit-BB (PDGF-BB), 200 µg/ml of ascorbic acid (AsA), 1 mg/ml of dexamethasone (DEX), and 5 µM of hydrogen peroxide (H_2O_2). Cell growth was measured after 6 days of treatment by staining the wells with crystal violet, a dye that stains DNA to have a direct visual assessment of cells after treatment. Then, crystal violet was solubilized with 10% acetic acid, and the absorbance was read at 562 nm, on the spectrophotometer.

Immunofluorescence: About 4,000 cells/well were seeded on coverslips in a 24-well plate. After 24 h, the cells were fixed in 4% paraformaldehyde in PBS (1X; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) for 10 min at room temperature. The cells were then washed with PBS 1X for 5 min, incubated with blocking solution (4% BSA in PBS 1X) at room temperature for 30 min, and finally, incubated with CD45 fluorochrome-conjugated antibody (2D1, 1:100 in 2% BSA) and phalloidin (1:40 in 2% BSA; BD, Franklin Lakes, NJ). The slides were washed with PBS and incubated with 4′,6-diamidino-2-phenylindole (DAPI; 1:10,000 in 2% BSA) for 10 min at room temperature. Finally, the slides were washed twice with PBS and mounted.

Real-time quantitative reverse transcription PCR (qRT-PCR): From five patients, vitreous samples were individually collected at the beginning of the 25 gauge pars plana from the central vitreous cavity and immediately centrifuged at 13,800 x g for 10 min. The clear supernatant was discarded, whereas the cellular component and debris (in situ vitreous cells) were immediately frozen at −20 °C until analysis. From six other patients, primary cultures were set up: The cells were seeded on six-multiwell plates in 10% FBS IMDM and cultured for 3 weeks (cultured vitreous cells). Then, the in situ and cultured vitreous cells were washed with PBS 1X, and total RNA was extracted with the Cells-to-CT™ 1-Step PowerSYBR® Green Kit (Ambion; Thermo Fisher Scientific, Waltham, MA) as previously described [19,20] and following the manufacturer's instruction. Twenty microliters of RNA were used for retrotranscription using the SensiFAST™ cDNA Synthesis Kit (Bioline, Aurogene Srl,

TABLE 1. OPHTHALMOLOGICAL CHARACTERISTICS OF THE RECRUITED PATIENTS.

N°	Age	Gender	Intervention	Reason for the intervention
1	73	F	VTC 25 G	Macular hole
2	81	M	VTC 25 G	Spinteropia
3	76	M	VTC 25 G	Macular pucker
4	77	F	VTC 25 G	Dislocated IOL
5	81	F	VTC 25 G	Retinal detachment
6	79	M	VTC 25 G	Retinal detachment on macular pucker
7	56	F	VTC 25 G+FACO+IOL	Spinteropia + macular pucker + cataract
8	40	M	VTC 25 G	Retinal atrophy
9	77	M	VTC 25 G	Macular pucker
10	75	F	VTC 25 G+FACO+IOL	Macular pucker+cataract
11	80	M	VTC 25 G	Macular pucker
12	70	M	VTC 27 G+FACO+IOL	Macular pucker+cataract
13	78	M	VTC 25 G+FACO+IOL	Macular pucker+cataract
14	77	F	VTC 27 G+FACO+IOL	Macular pucker+cataract
15	52	M	VTC 25 G	Retinal detachment
16	66	M	VTC 25 G+FACO+IOL	Diabetic retinopathy+cataract
17	74	F	VTC 25 G+FACO+IOL	Retinal detachment+cataract
18	75	M	VTC 27 G	Macular pucker
19	54	F	VTC 25 G+FACO+IOL	Macular hole+cataract
20	52	M	VTC 25 G+FACO+IOL	Macular hole+cataract
21	79	F	VTC 27 G	Macular pucker
22	66	F	VTC 25 G	Diabetic retinopathy
23	55	M	VTC 25 G+FACO+IOL	Retinal detachment+cataract
24	63	M	VTC 25 G	Macular pucker
25	78	M	VTC 25 G	Macular pucker
26	52	F	VTC 25 G+FACO+IOL	Retinal detachment+cataract
27	87	M	VTC 25 G+FACO+IOL	Macular pucker+cataract
28	66	M	VTC 25G	Macular pucker
29	74	F	VTC 25 G+FACO+IOL	Macular pucker+cataract
30	83	F	VTC 25 G	Macular pucker
31	85	M	VTC 25 G	Retinal detachment
32	82	M	VTC 25 G	Macular pucker+cataract
33	86	M	VTC 25 G	Macular pucker+cataract

N°	Age	Gender	Intervention	Reason for the intervention
34	78	M	VTC 25 G	Spinteropia+cataract
35	70	M	VTC 27 G+FACO+IOL	Macular pucker+cataract
36	64	F	VTC 25 G	Retinal detachment
37	69	F	VTC 25 G+FACO+IOL	Retinal detachment+cataract
38	65	M	VTC 25 G+FACO+IOL	Macular hole+cataract
39	41	F	VTC 25 G+FACO+IOL	Retinal detachment+cataract+macular pucker
40	70	F	VTC 25 G	Macular hole
41	70	F	VTC 25 G+FACO+IOL	Retinal detachment+cataract
42	70	M	VTC 25 G	Retinal detachment
43	48	F	VTC 25 G	Diabetic retinopathy
44	65	M	VTC 25 G	Macular pucker
45	55	M	VTC 25 G	Retinal detachment
46	70	F	VTC 25 G	Macular hole
47	68	M	VTC 25 G	Macular pucker
48	66	F	VTC 25 G+FACO+IOL	Retinal detachment+cataract
49	75	F	VTC 25 G	Macular hole
50	68	M	VTC 25 G	Macular pucker
51	73	F	VTC 25 G	Macular hole
52	63	M	VTC 25 G+FACO+IOL	Retinal detachment+cataract
53	74	F	VTC 25 G	Macular pucker
54	70	M	VTC 25 G	Retinal detachment

List of patients randomly chosen among those undergone a 25 gauge or 27 gauge pars plana vitrectomy from the central vitreous cavity for vitreoretinal disorders. VTC 25 G: 25 gauge vitrectomy; VTC 27 G: 27 gauge vitrectomy; IOL.: intra ocular lens; FACO: phacoemulsification.

TABLE 2. DETAILS OF THE PRIMERS USED FOR qRT-PCR TECHNIQUE.

Gene	Forward primer (5'-3')	Reverese primer (5'-3')	References
hCD45	CTGACATCATCACCTAGCAG	TGCTGTAGTCAATCCAGTGG	[37]
hCD11a	TGAGAGCAGGCTATTTGGGTTAC	CGGCCCATGTGCTGGTAT	[38]
hCD64	GCCACAGAGGATGGAAATGT	CATGAAACCAGACAGGAGTGG	[39]
hCD163	TCAGAGTTTCAACCAGAAGGAG	TGTCATCTGAGGTTCTTGTCC	[40]
hCD68	TGGATTCATGCAGGACCTCC	CGCCATGTAGCTCAGGTAGACA	[41]
hCD11b	GGCCATGCACAGATACCAGGT	CTGGGGGTGCGATTTTCT	[41]
hCD16	TCGAGCTACTTCATTGACGC	GATATGGACTTCTAGCTGCACC	[42]
H vimentin	AGGAAATGGCTCGTCACCTTCGTGAATA	GGAGTGTCGGTTGTTAAGAACTAGAGCT	[43]
hGFAP	ACATCGAGATCGCCACCTAC	ACATCACATCCTTGTGCTCC	[44]
hRlbp	GCTGCTGGAGAATGAGGAAACTC	GGCTGGTGGATGAAGTGGAT	[45]
B2M	AGCAAGGACTGGTCTTTCTATCTC	ATGTCTCGATCCCACTTAACTA	[46]

Abbreviations: hGFAP, human glial fibrillary acidic protein; hRlbp, human retinaldehyde binding protein 1; B2M, beta-2-Microglobulin used as reference gene to normalize the cDNA in different samples; qRT-PCR, quantitative real-time polymerase chain reaction.

Rome, Italy) at 42 °C for 15 min and 85 °C for 5 min. cDNAs were stored at −80 °C until PCR analysis. PCR primers were designed using NCBI/Primer-BLAST and synthesized by Sigma-Merck (Merck Life Science S.r.l., Milan, Italy). The SensiFAST™ SYBR® No-ROX Kit (Bioline, Aurogene Srl) was used to perform qRT-PCR following the manufacturer's protocol: 1 cycle at 95 °C for 2 min, 40 cycles at 95 °C for 5 s, 1 cycle at 60 °C for 30 s, and 1 cycle at 95 °C for 1 min. The PCR primers (Table 2) were designed using NCBI/Primer-BLAST and synthesized by Sigma-Merck (UK). Gene expression analysis was performed with CFX Manager software (BioRad, Hercules, CA). Each reaction was performed in triplicate. The specificity of the PCRs was confirmed with melt curve analysis. Nonspecific amplifications were never detected.

HA assay: To perform the HA assay, 5,000 hyalocytes/well were seeded in 48-well plates containing 0.5 ml IMDM (+ 10% FBS) and stimulated for 6 days with different agents: 10 ng/ml of bFGF, 5 ng/ml of TGF-β, 10 ng/ml of PDGF-BB, 200 µg/ml of AsA, 1 mg/ml of DEX, and 5 µM of H_2O_2. HA levels were detected in 10 µl of primary culture supernatant using the hyaluronic acid (human) AlphaLISA® immunoassay kit (PerkinElmer, Waltham, MA). Two different types of cells, lung adenocarcinoma (A549) cells (ATCC, LGC Standards S.r.l., Milan, Italy), were used for comparison purposes based on their different HA production in 6 days [21,22]. HA levels were measured with an EnVision plate reader (PerkinElmer), and the values are expressed as ng HA/ng cell protein.

Statistical analysis: Statistical analysis of the data was performed using analysis of variance (ANOVA) with Tukey's post-hoc correction. A p value of less than 0.05 was considered statistically significant. All data were expressed as mean ± standard deviation (SD).

RESULTS

Optimization of hyalocyte culture conditions: From 54 vitrectomy samples representative of different eye diseases, 54 different cell cultures were set up in about 6 weeks as described in the Method section, and different growth media were tested, using collagenase treatment or not, to allow the setting of primary cultures. Samples subjected to collagenase treatment showed low viability which did not allow the establishment of primary cultures. IMDM supplemented with serum turned out to be the best growth condition for samples that were directly seeded in collagen-coated plates. Like most primary cultures, these cells were not immortalized, but they were able to grow in vitro for 1–3 months. The cultures contained cells showing two different morphologies: elongated (or spherical), with a few stout processes (Figure 1A), or stellate, with some short processes (Figure 1B).

To confirm that the cells growing in culture were hyalocytes, we performed immunofluorescent staining with antibodies recognizing CD45. As shown in Appendix 1, the immunofluorescence detection demonstrated that these cells expressed the CD45 antigen [9,13]. Moreover, qRT-PCR was performed to check the presence of CD45, CD11a, CD64, and CD163 and the absence of CD68 and CD11b in the in situ and in cultured vitreous cells. There were no significant differences in the expression of the hyalocyte mRNA markers between the in situ and cultured cells (Figure 2), demonstrating that the primary cultures retained the characteristics of the cells extracted from the tissue. Importantly, the
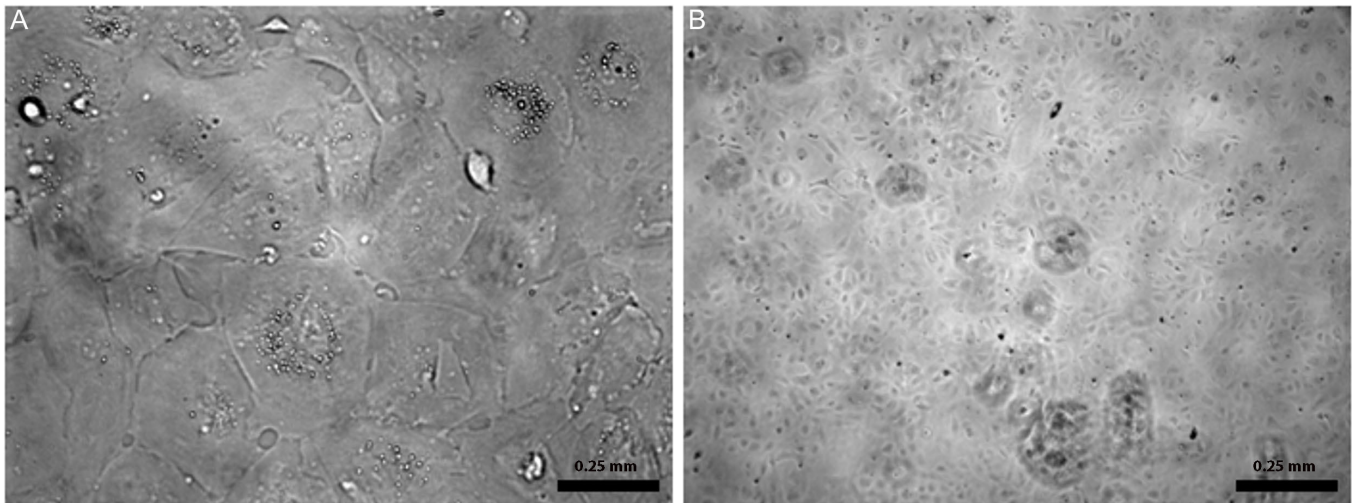
Figure 1. In vitro primary cultures microphotographies. Cells show two main morphologies: elongated (or spherical), with a few stout processes (**A**), or stellate, with some short processes (**B**). Scale bar: 0.25 mm.

primary cultures of hyalocytes did not contain any contaminating cell types as mRNA expression of CD68 and CD11b was not detected (Figure 2), In further analysis, we could not find amplification products for CD16, vimentin, GFAP, and Rlbp, chosen as the typical markers for macrophages, neutrophils, fibroblasts, and retinal glial and human RPE cells (data not shown), respectively, suggesting the absence of contaminating cells in the primary cultures of hyalocytes.

Moreover, as hyalocytes are known to produce HA [23], we investigated the accumulation of this molecule in the supernatant of the cell cultures. As expected, we detected the presence of HA (0.861 0.06 ng/ng cellular protein; Figure 3), further sustaining that the primary cultures were enriched in hyalocytes. Overall, the data show that we were able to set up

primary cultures of cells that display morphological, immunogenic, and functional characteristics typical of hyalocytes.

Biologic effects of treatments: To understand whether the environmental conditions could modify the growth and the shape of the cells, as well as their biologic activity, we exposed the cultures to different agents known to affect non-human hyalocytes [3,24,25]. One of the aims of this study was to compare the difference between human and animal hyalocytes, to better understand the role of human hyalocytes in the pathogenesis of different ocular diseases. In the experiments, we tested the effects of 10 ng/ml bFGF, 5 ng/ml TGF-β, 10 ng/ml PDGF-BB, 200 μg/ml AsA, 1 mg/ml DEX, and 5 μM H_2O_2.
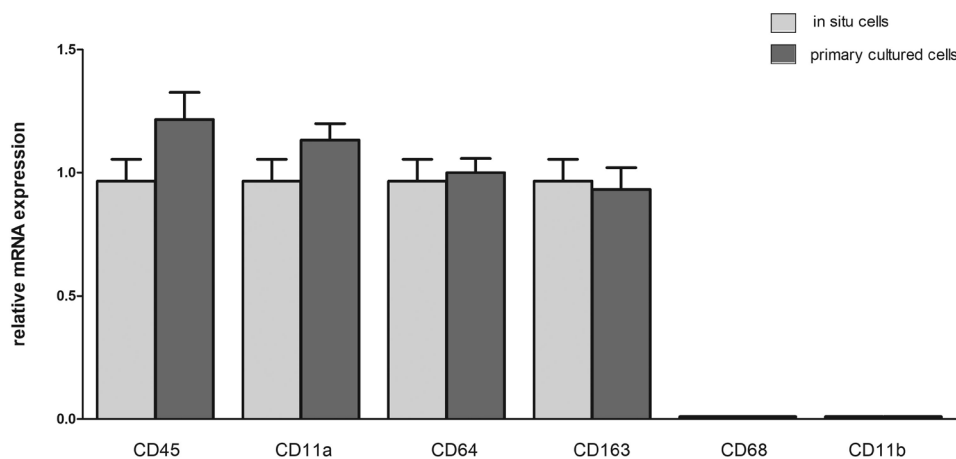

Figure 2. qRT-PCR assay. Relative mRNA expression of CD45, CD11a, CD64, CD163, CD68, and CD11b in situ *v*itreous cells and in cultured vitreous cells. Measurements were performed in triplicate.
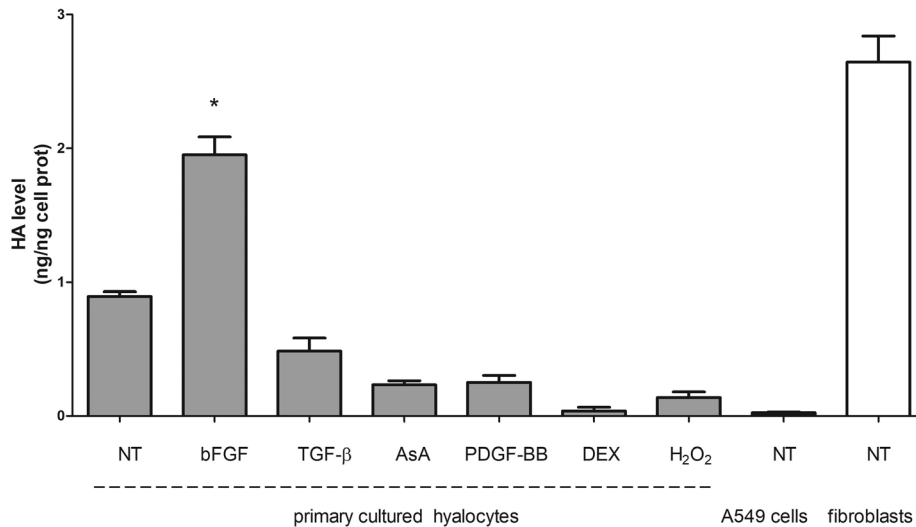
Figure 3. Hyaluronic acid assay. Hyaluronic acid was quantified in supernatants from primary culture hyalocytes after 6 days of treatment with 10 ng/ml basic fibroblast growth factor (bFGF), 5 ng/ml transforming growth factor beta 1 (TGF-β), 10 ng/ml platelet-derived growth factor subunit-BB (PDGF-BB), 200 μg/ml ascorbic acid (AsA), 1 mg/ml dexamethasone (DEX) and 5 μM H2O2., and in media from A549 cells and human fibroblasts. Measurements were performed in triplicate; data were represented as means ± standard deviation (SD); The analysis of variance (ANOVA) with Tukey's post-hoc correction was performed. *$p < 0.001$ versus untreated (NT) primary cultured hyalocytes.

To assess the effects of the treatments on cell shape and cytoskeletal filament organization, hyalocytes were stained with phalloidin. As shown in Figure 4, the untreated hyalocytes showed a filamentous actin cytoskeleton organized in numerous but thin stress fibers (Figure 4A). Lamellipodia were observed after 5 days of treatment with bFGF (Figure 4B), suggesting an increased cell motility. TGF-β (Figure 4C) and PDGF-BB (Figure 4D) induced actin cytoskeleton reorganization in long and thick actin stress fibers. The supplementation with AsA inhibited actin polymerization and induced the appearance of small and rounded cells (Figure 4E). DEX (Figure 4F) induced actin fiber organization in two different patterns: short stress fibers and thick filamentous fibers close to the cell membrane. Finally, H_2O_2 caused the appearance of thick filamentous actin fibers close to the cell membrane (Figure 4G).

We also evaluated the proliferation of hyalocytes in the same experimental conditions. As shown in Figure 5, the increase in cell number was clear upon the supplement of 10 ng/ml of bFGF versus untreated cells (NT; resulting in a threefold increase in the proliferation rate). The TGF-β, PDGF-BB, DEX, and H_2O_2 treatments had no statistically significant effect on cell proliferation.

As the identification of agents that could increase HA production is important for their possible pharmacological application for vitreous regeneration, we evaluated the effect of these substances on HA production. As shown in Figure 3, the level of hyaluronic acid in the cellular supernatant was statistically significantly increased after treatment with bFGF (1.95 0.08 ng/ng cellular protein), whereas the other treatments did not modify production of hyaluronic acid.

DISCUSSION

Vitreous has long been considered an inert fluid with the only function of keeping the retina adherent to the posterior eye's surface. Recently, many researchers have examined vitreous properties and functions to understand whether they could play a role in the pathogenesis of some ocular diseases. Although the presence of cells inside the vitreous has been known for more than a century, their role has remained unknown for many decades, and even today, it is uncertain. Nevertheless, much evidence shows that hyalocytes are involved in the different ocular pathogenesis. The interaction between an inflammatory environment and hyalocytes could stimulate these cells to change their behavior and gain contractile function [15,24]. Due to this new feature, hyalocytes may pull the internal limiting membrane of the retina, causing the formation of macular pucker or macular hole [8,13-16]. Schumann et al. [13] showed that there is a positive correlation between macular hole size and cell density at the internal limiting membrane. However, the proliferation of hyalocytes could represent one of the possible ways to obtain vitreous regeneration. Although synthetic vitreous substitutes are still the only choice after vitrectomy, supplementation with bFGF during the expansion of porcine hyalocytes appears to be a promising approach to gain enough cells within a short time frame useful for regenerating human vitreous [3].
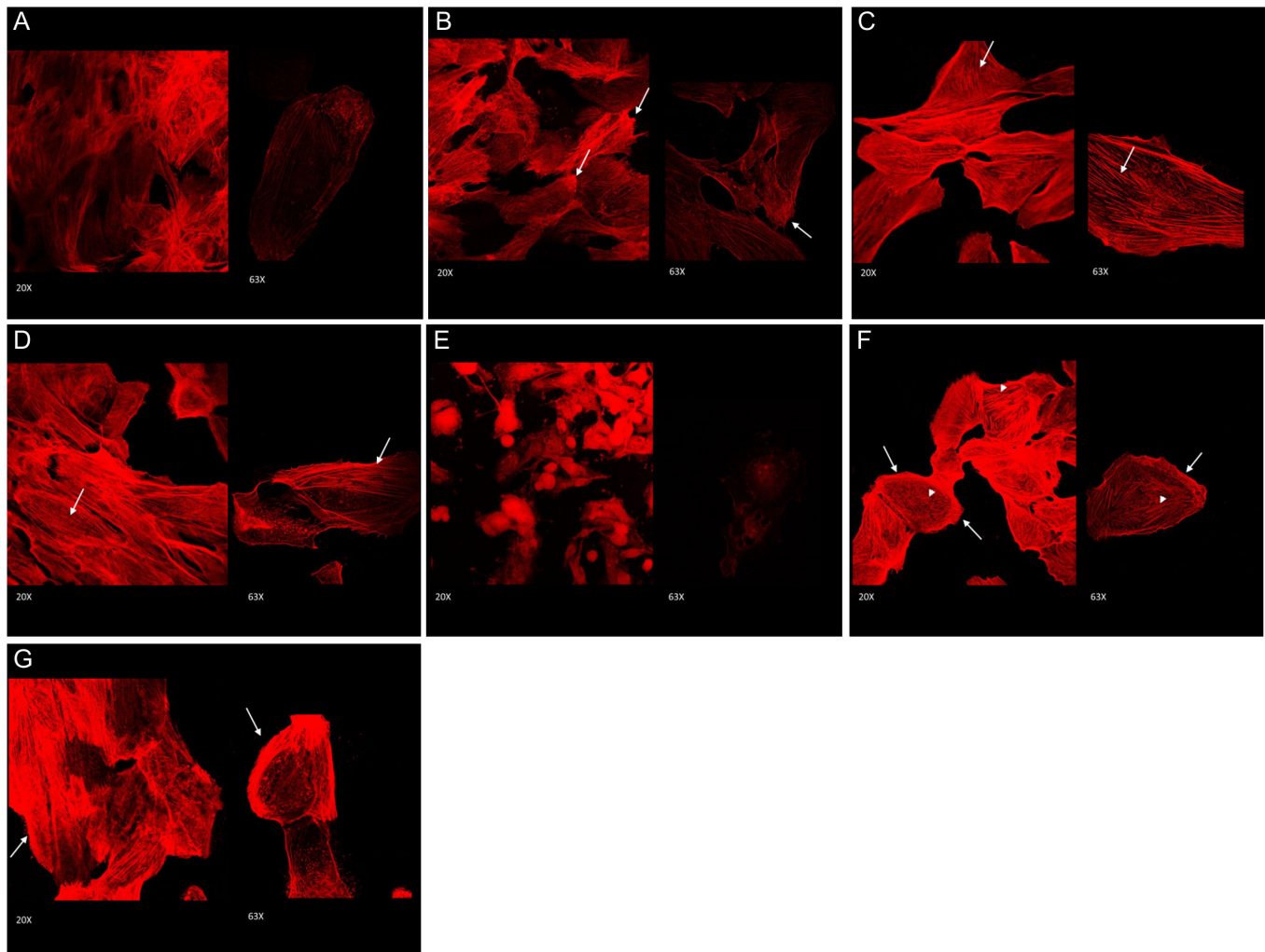
Figure 4. Immunofluorescent staining. Hyalocytes were stained with phalloidin, showing the cell shape and cytoskeletal changes after 6 days of exposure to the different treatments (doses are indicated in the Methods section). **A**: Control. **B**: Basic fibroblast growth factor (bFGF). **C**: Transforming growth factor beta 1 (TGF-β). **D**: Platelet-derived growth factor subunit-BB (PDGF-BB). **E**: Ascorbic acid (AsA). **F**: Dexamethasone (DEX). **G**: H_2O_2.

In this context, based on the existing studies on animal hyalocytes, the availability of cultures of human hyalocytes to investigate treatments affecting their growth and biologic properties is necessary. For the first time, this study provided a reproducible experimental technique for isolating and growing human hyalocytes in primary culture. Several growth conditions were tested to improve the rate of success in establishing primary cultures. In our experience, direct plating on collagen-coated dishes turned out to be more effective than collagenase pretreatment of cells derived from surgical specimens. Moreover, among the tested media, IMDM with the addition of FBS provided the best results. In these conditions, we obtained primary cultures that remained alive for several months. Previous studies on hyalocytes were clearly different from ours. Researchers were able to obtain

hyalocyte cell lines in active proliferation only from animals, whereas the few studies conducted on human cells have allowed only to document the presence of hyalocytes in the humor vitreous and to study their morphology [8,9,14,17,26]. Once we were able to generate hyalocyte cultures, we investigated their behavior in the presence of different stimulations. bFGF is involved in cell survival activities, mitogenic processes, embryonic development, cell growth, morphogenesis tumor growth, and invasion [27]. Although it has been shown that bFGF can inhibit the differentiation of embryonic stem cells, and it is necessary for the cells to remain in an undifferentiated state [27], at the same time, bFGF, in conjunction with the bone morphogenetic protein 4 (BMP4), promotes differentiation of stem cells to mesodermal lineages [28]. In agreement with the study by Sommer et al. [3] on
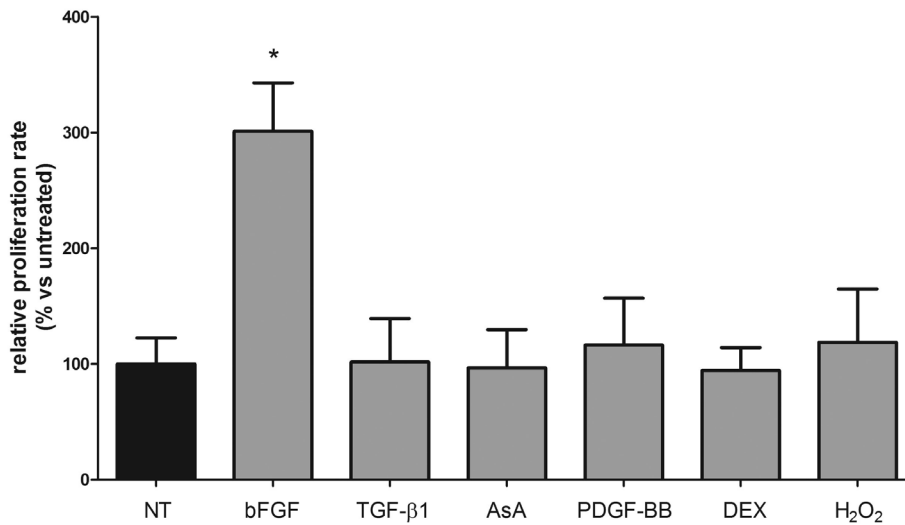
Figure 5. Crystal violet staining. Cell proliferation was assessed after 6 days of treatment with 10 ng/ml basic fibroblast growth factor (bFGF), 5 ng/ml transforming growth factor beta 1 (TGF-β), 10 ng/ml platelet-derived growth factor subunit-BB (PDGF-BB), 200 µg/ml ascorbic acid (AsA), 1 mg/ml dexamethasone (DEX), and 5 µM H_2O_2. Measurements were performed in triplicate; data were represented as % versus untreated (means ± standard deviation, SD). The analysis of variance (ANOVA) with Tukey's post-hoc correction was performed. *p<0.001 versus untreated (NT) primary cultured hyalocytes.

porcine hyalocytes, the present experiments revealed that bFGF is endowed with growth-promoting activity, increases HA production, and induces morphological changes compatible with a motile phenotype, suggesting that bFGF could stimulate hyalocytes to gain new functions and a different behavior which could be related to the pathogenesis of various ocular diseases.

TGF-β could be one of the molecules produced by hyalocytes that have a role in the modulation of the retinal microenvironment. Sommer et al. showed that TGF-β inhibits the proliferation of porcine hyalocytes [3]; however, in this study we did not observe differences in cell growth upon TGF-β treatment. The difference between these results could be ascribable to the intrinsic difference between human and porcine hyalocytes. As the acquisition of contractile functions of hyalocytes is mandatory for the pathogenesis of macular pucker [8,15,16], we investigated the effects of TGF-β on actin cytoskeleton. In agreement with Sommer et al. [3], we observed that TGF-β increased the thickness of the actin filaments within the cells.

Another difference between our and Sommer's work is related to AsA. In their study, AsA clearly stimulated the proliferation of porcine hyalocytes [29]. Noda et al. [24] demonstrated that bovine hyalocytes express platelet-derived growth factor α and β (PDGF-α and PDGF-β) receptors, whose activation promotes cell growth and migration.

Another important aspect that could emerge from this study is the possibility of exploiting the novel cell culture protocol to investigate the interaction between hyalocytes and other cell lines. Nuzzi et al. [30] analyzed the interaction between vitreous environment, cultured RPE (ARPE-19) cells, and mesenchymal stems cells (MSCs). ARPE-19 cells could modify the effect of different agents on human hyalocytes. In this context, it could be interesting to study, proving a reproducible experimental model, the interaction between human hyalocytes, ARPE-19, and different drugs. Nuzzi et al. demonstrated that MSCs are sensible to corticosteroid and to ARPE-19 cells. Moreover, DEX inhibits the proliferation of human bone marrow MSCs, and the addition of RPE cells decreases the toxicity of DEX for MSCs. Future studies could investigate the role of MSCs in the metabolism of hyalocytes. This interaction could be important for treating age-related macular degeneration (AMD) [31]. Knowing the interaction between MSCs, hyalocytes, ARPE-19, and different agents like DEX is essential to improve the treatment of AMD and other ocular diseases [30,31].

Altogether, the present results show that although human hyalocytes share some characteristics with animal hyalocytes, human hyalocytes have their own features typical of the species, confirming how important the human experimental model for hyalocytes is for investigating human pathologies and their treatments. The novelty of this study was a protocol for generating primary hyalocyte cultures to study the growth, cytoskeleton changes, and HA production in response to different agents.

Novel approaches for vitreous replacement are needed to improve clinical therapy after vitrectomy. After

vitreous-retinal surgery, it is necessary to use a vitreous substitute that has the functions of ensuring retinal adherence, avoiding intraocular hemorrhage, and maintaining correct eye tone [32,33]. Although some substances like silicon have these features and can be allowed inside the eye for several days after surgery, all these vitreous substitutes can cause side effects, such as glaucoma, cataract, and retinal toxicity [32-34]. For these reasons, several researchers have investigated the possibility of generating new materials to substitute the human vitreous. In addition to studies on hydrogels and natural polymers, studies on the regeneration of human vitreous are extremely promising [34-36].

To proceed in this direction, it is essential to improve our knowledge of the vitreous and its cell physiology. In this context, the possibility of isolating and growing human hyalocytes represents a big step forward, in particular for further investigations of potential positive or negative effects different growth factors.

APPENDIX 1. IMMUNOFLUORESCENT STAINING (GREEN SIGNAL) WITH ANTIBODY RECOGNIZING THE LEUKOCYTE COMMON ANTIGEN (CD45).

To access the data, click or select the words "Appendix 1."

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