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Intestinal Ellagitannin Metabolites Ameliorate Cytokine-Induced Inflammation and Associated Molecular Markers in Human Colon Fibroblasts

Juan A. Giménez-Bastida, Mar Larrosa, Antonio González-Sarrías, Francisco Tomás-Barberán, Juan C. Espín, and María-Teresa García-Conesa*

Research Group on Quality, Safety and Bioactivity of Plant Foods, Department of Food Science and Technology, CEBAS-CSIC, P.O. Box 164, 30100 Campus de Espinardo, Murcia, Spain

Supporting Information

ABSTRACT: Pomegranate ellagitannins (ETs) are transformed in the gut to ellagic acid (EA) and its microbiota metabolites, urolithin A (Uro-A) and urolithin B (Uro-B). These compounds exert anti-inflammatory effects in vitro and in vivo. The aim of this study was to investigate the effects of Uro-A, Uro-B, and EA on colon fibroblasts, cells that play a key role in intestinal inflammation. CCD18-Co colon fibroblasts were exposed to a mixture of Uro-A, Uro-B, and EA, at concentrations comparable to those found in the colon (40 μM Uro-A, 5 μM Uro-B, 1 μM EA), both in the presence or in the absence of IL-1β (1 ng/mL) or TNF-α (50 ng/mL), and the effects on fibroblast migration and monocyte adhesion were determined. The levels of several growth factors and adhesion cytokines were also measured. The mixture of metabolites significantly inhibited colon fibroblast migration (~70%) and monocyte adhesion to fibroblasts (~50%). These effects were concomitant with a significant down-regulation of the levels of PGE_2, PAI-1, and IL-8, as well as other key regulators of cell migration and adhesion. Of the three metabolites tested, Uro-A exhibited the most significant anti-inflammatory effects. The results show that a combination of the ET metabolites found in colon, urolithins and EA, at concentrations achievable in the intestine after the consumption of pomegranate, was able to moderately improve the inflammatory response of colon fibroblasts and suggest that consumption of ET-containing foods has potential beneficial effects on gut inflammatory diseases.

KEYWORDS: *urolithins, ellagic acid, cell adhesion, cell migration, chemokines, growth factors, microbiota metabolites*

INTRODUCTION

The two major forms of inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are chronic remittent or progressive inflammatory conditions that may affect the entire gastrointestinal tract or the colonic mucosa, respectively, and are associated with an increased risk for colon cancer.[1] The incidence of IBD continues to rise in both the developed and developing worlds, indicating that "Westernization" may be conditioning the expression of these disorders and that environmental factors play a significant part in IBDs.[2] Of these, the diet is likely to have an important as yet poorly defined role on the development and progression of intestinal inflammatory diseases, with some dietary constituents contributing to the disease but others protecting against it.[3] In particular, the consumption of fruits, vegetables, olive oil, grains, and nuts has been inversely associated with CD.[4] All of these plant-derived foods are rich in polyphenols, which have been extensively reported to have anti-inflammatory properties.[5] Because a large proportion of the dietary polyphenols are not absorbed and these compounds and (or) their microbiota metabolites accumulate in the intestine, the effects of these compounds against intestinal inflammation have been investigated using animal models of colitis and in intestinal cells treated with pro-inflammatory cytokines. These studies provide evidence that polyphenols can effectively modulate intestinal inflammation.[6]

More specifically, a pomegranate extract (PE) rich in ellagitannins (ETs) has been shown to have anti-inflammatory properties in a rat model of induced colitis by regulating the expression of genes involved in the inflammatory pathways, decreasing inflammatory markers and preserving the colon epithelium architecture.[7] ETs release ellagic acid (EA) in the gut, and both, ETs and EA, are poorly absorbed in the stomach and small intestine and largely metabolized by unidentified bacteria in the intestinal lumen to produce urolithins (dibenzopyranones), mostly urolithin A (Uro-A) and urolithin B (Uro-B),[8] which can be found at relatively high concentrations in the colon (micromolar levels).[7,9] The intestinal anti-inflammatory properties of PE may be attributed to the urolithins, in particular Uro-A, which has also been reported to exert anti-inflammatory activity in vivo[7] and in cell models of human colon myofibroblasts[10] and of aortic endothelial cells.[11]

Pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-α) and interleukin 1-beta (IL-1β) are essential in mediating the inflammatory response, causing a disturbance in the intestinal barrier and increasing tissue penetration of luminal antigens. Inhibition of the cytokine-induced increase in intestinal permeability has an important protective effect against

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intestinal mucosal damage and development of intestinal inflammation.[12] Colonic subepithelial myofibroblasts, which reside just beneath the epithelial layer, form part of the gut barrier. They are critically involved in wound healing and intestine mucosa repair because of their ability to modulate extracellular matrix components (ECM).[13] They also play an active role in the immune response of the intestine. Upon activation, these cells produce soluble cytokines, chemokines, adhesion proteins, and growth factors and initiate the recruitment of immune cells to the site of tissue injury and inflammation.[14]

With a view to further elucidating some of the putative mechanisms by which dietary ETs may contribute to protect against intestinal inflammation, in the present study, we investigated some of the cellular and molecular responses associated with the exposure of cytokine-activated human colon fibroblasts (CCD-18Co) to a mixture of Uro-A, Uro-B, and EA at concentrations representative of those that may be found in vivo in the colon after the dietary intake of ET-containing foods. For comparative purposes, we also examined the effects of the same levels of each of the individual compounds. We specifically explored the effects of these ET metabolites on (i) colon fibroblasts migration, (ii) THP-1 monocyte adhesion to colon fibroblasts, and (iii) molecular markers involved in migration (growth factors) and adhesion (adhesion proteins).

MATERIALS AND METHODS

Materials. Uro-A (3,8-dihydroxy-6*H*-dibenzo(*b*,*d*)pyran-6-one) and Uro-B (3-hydroxy-6*H*-dibenzo(*b*,*d*)pyran-6-one) were chemically synthesized by Kylolab S.A. (Murcia, Spain). EA, calcein-AM, human recombinant TNF-α, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate-buffered saline (PBS) was from Fisher Scientific (USA). IL-1β was obtained from Calbiochem (Darmstadt, Germany). DMSO was from Panreac (Barcelona, Spain). Ultrapure Millipore water was used for all solutions.

Cell Culture. Human acute monocytic THP-1 cells were obtained from the European Collection of Cells Cultured (ECACC) (Salisbury, U.K.) and maintained in RPMI 1640 culture medium containing 10% v/v fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Invitrogen S.A., Barcelona, Spain) and maintained at 37 °C under a 5% CO_2/95% air atmosphere at constant humidity. Cells passages between 15 and 30 were used for the experiments. The myofibroblast-like cell line CCD-18Co was obtained from the American Type Culture Collection (ATCC; ref CRL-1459) (Rockville, MD, USA). Unless otherwise stated, cells were routinely grown in Eagle's minimum essential medium (EMEM) containing 10% v/v FBS and supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (Gibco, Invitrogen S.A.) and maintained at 37 °C under a 5% CO_2/95% air atmosphere at constant humidity. Cell cultures between 16 and 19 population doubling levels (PDLs) were used for all experiments.

Prostaglandin E_2 (PGE_2) Analysis. Confluence colon fibroblasts seeded in 96-well plates were washed twice with PBS and incubated in medium (0.1% FBS) for 24 h. Next, the cells were treated with IL-1β (1 ng/mL) or TNF-α (50 ng/mL) alone or in combination with each of the tested metabolites dissolved in DMSO ((i) Uro-A (40 μM), (ii) Uro-B (5 μM), (iii) EA (1 μM), and (iv) a mixture of these molecules (40 μM Uro-A, 5 μM Uro-B, and 1 μM EA; MIX)) for 18 h (time point at which maximum production of PGE_2 is observed).[15,16] Control cells were treated in parallel with the equivalent amount of DMSO (0.5% v/v). The culture medium was then removed and frozen at −80 °C until analysis. PGE_2 levels were measured using an immunoenzymatic method (Cayman Chemicals, San Diego, CA, USA) according to the manufacturer's instructions.

Migration Assay. Colon fibroblast migration was examined using the procedure described by Giménez-Bastida et al.[11] with some modifications. CCD18-Co confluent monolayers were then changed into fresh medium containing 0.1% FBS for 24 h and a group of cells destroyed or displaced by scratching a line horizontally through the monolayer with a sterile pipet tip. Media, dislodged cells, and debris were washed twice with PBS before the culture medium was replaced by fresh medium (0.1% FBS). Next, the cells were treated with each of the inflammatory cytokines, IL-1β (1 ng/mL) or TNF-α (50 ng/mL), alone or in combination with each of the tested metabolites dissolved in DMSO ((i) Uro-A (40 μM), (ii) Uro-B (5 μM), (iii) EA (1 μM), and (iv) a mixture of these compounds (40 μM Uro-A, 5 μM Uro-B, and 1 μM EA; MIX)) for 48 h. Control cells were treated in parallel with the equivalent amount of DMSO (0.5% v/v) . The open gap was inspected over time as the cells moved in and filled the damaged area using a phase contrast inverted microscope. Randomly selected views along the scraped line were photographed on each well using a charge-coupled device (CCD) Nikon Digital Eclipse DXM1200 camera (Nikon, Inc., Tokyo, Japan) attached to the microscope after the treatment (time 0) and following 48 h of exposure. The average distance between the two sides of the gap was determined by measuring the distance (μm) between two points (n = 10 measurements) along each photographed area. The final migrated distance was calculated as the difference between the gap distance at time 0 and after 48 h of incubation. Data are representative of three experiments (n = 2 wells per treatment).

Cell Viability Assay. Cell viability was estimated using the MTT assay. This assay determines total mitochondrial activity, which is related to the number of viable cells and used to determine in vitro cytotoxic effects.[17] Cells were treated following the same protocol described for the migration assay. Following 48 h of incubation, the culture medium was removed and the cells were washed twice with sterile PBS. Then, 1 mL of MTT solution (1.0 mg/mL in FBS-deprived medium) was added to the cells and incubated for a further 4 h. The formazan crystals formed in the viable cells were solubilized with 625 μL of DMSO, and the optical density was measured at a test wavelength of 570 nm and a reference wavelength of 690 nm using a microplate reader (Fluostar Galaxy, BMG Lab. Technologies v5.0). Data are presented as the mean values ± SD from three independent experiments (n = 2 wells per experiment).

Cell Adhesion Assay. Monocyte adhesion to colon fibroblasts was evaluated using the human leukemia monocytic THP-1 cells as previously described.[11] Cultured monocytes were resuspended in PBS (1×10^6 cells/mL) and labeled with calcein-AM (5 μM final concentration) for 30 min at 37 °C. Cells were washed twice with PBS before addition to the fibroblasts. Confluent colon fibroblasts cells cultured in 96-well plates were co-incubated with freshly prepared IL-1β (1 ng/mL) or TNF-α (50 ng/mL), alone or in combination with each of the tested metabolites dissolved in DMSO ((i) Uro-A (40 μM), (ii) Uro-B (5 μM), (iii) EA (1 μM), and (iv) a mixture of these molecules (40 μM Uro-A, 5 μM Uro-B, and 1 μM EA; MIX)) for 12 h. Control cells were treated in parallel with the equivalent amount of DMSO (0.5% v/v). After treatment, the fibroblasts were washed twice with PBS and co-incubated with the calcein-labeled monocytes (2×10^5 cells per well) in the dark for 1 h at 37 °C. Nonadhering cells were removed and the remaining cells washed twice with PBS before fluorescence was measured with a fluorescence-detecting microplate reader (Fluostar Galaxy, BMG Lab. Technologies v5.0) using excitation at 492 nm and emission at 520 nm. Experiments were carried out in triplicate (n = 6 wells per treatment).

Measurement of Growth Factors, Adhesion Molecules, and Cytokines by ELISA. CCD18-Co colon-fibroblast confluent cells were treated with the pro-inflammatory cytokines IL-1β (1 ng/mL) or TNF-α (50 ng/mL) alone or in combination with Uro-A (40 μM) or the mixture of metabolites (40 μM Uro-A, 5 μM Uro-B, and 1 μM EA; MIX) in 0.1% FBS (v/v) culture media for 48 h (growth factors) or in 10% FBS (v/v) culture media for 12 h (adhesion proteins). After treatment, the culture medium was removed and frozen at −80 °C until analysis. The cells were washed twice with PBS and lysed with 0.5 mL of ice-cold lysis RIPA buffer containing a cocktail of protease inhibitors (Roche, Mannhein, Germany). Cell lysates were centrifuged at 23200g for 15 min at 4 °C and frozen at −80 °C until analysis. The

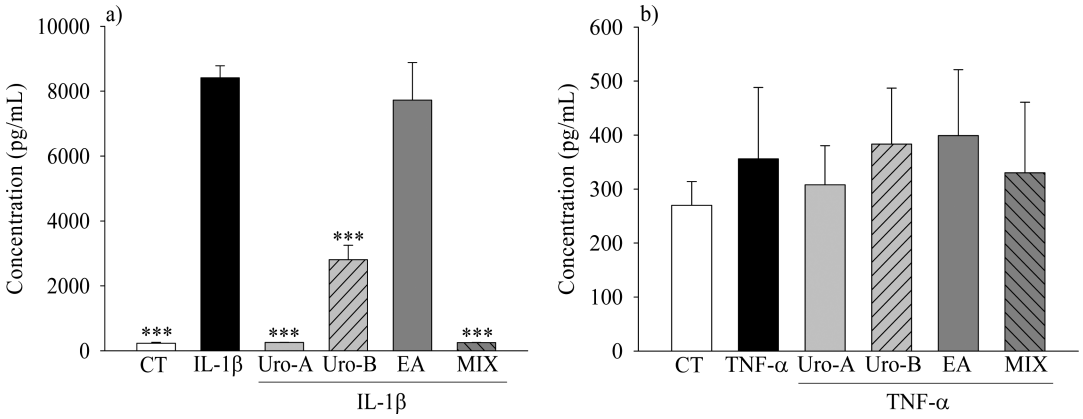

Figure 1. Levels of PGE_2 in the supernatants from CCD18-Co colon fibroblasts exposed to (a) IL-1β (1 ng/mL) or (b) TNF-α (50 ng/mL), alone or in combination with Uro-A (40 μM), Uro-B (5 μM), EA (1 μM), or a mixture of these molecules (40 μM Uro-A, 5 μM Uro-B, and 1 μM EA; MIX) for 18 h. Data are presented as the mean value from three independent experiments ± SD. Symbols indicate differences from the cytokine-treated samples; ***, $P < 0.001$.

protein content of the cell lysates was measured by the *DC* colorimetric assay at 750 nm (Bio-Rad, Barcelona, Spain) using a microplate reader (Infinite M200, Tecan, Grodig, Austria) and based on a BSA standard curve. Cell lysates were used to analyze specific molecules using the following commercially available human ELISA kits: platelet-derived growth factor β polypeptide (PDGF-BB; Prepotech, Rocky Hill, NJ, USA), β-type platelet-derived growth factor receptor (PDGF-R-β; Sino Biological, Schilde, Belgium), s-ICAM-1 (Bender MedSystems, Vienna, Austria), and VCAM-1 (Gen-Probe, San Diego, CA, USA). The minimum detection levels were 62 and 62.5 pg/mL for PDGF-BB and PDGF-R-β, respectively, and 6.25 and 0.6 ng/mL for s-ICAM-1 and VCAM-1, respectively. The protein content of the cell lysates was measured by the *DC* colorimetric assay at 750 nm (Bio-Rad) using a microplate reader (Infinite M200, Tecan) and based on a BSA standard curve. Culture medium was used for the analysis of interleukin-8 (IL-8), interleukin-6 (IL-6), plasminogen activator inhibitor-1 (PAI-1), and monocyte chemotactic protein-1 (MCP-1 or CCL2) using commercially available ELISA kits from Peprotech (Rocky Hill, NJ, USA). The minimum detection levels were 8 pg/mL for IL-8 and MCP-1, 32 pg/mL for IL-6, and 23 pg/mL for PAI-1. Analysis of the intensity related to the concentration was measured using a microplate reader (Infinite M200, Tecan). Data are presented as the mean value from three to six independent experiments ± SD.

Human Antibody Arrays. Changes in the expression levels of proteins involved in cell migration and cell adhesion were investigated using (i) human antibody membrane arrays RayBio Human Growth Factor Array, which contains 41 growth factors, and (ii) custom-designed human antibody arrays containing 20 markers of adhesion (RayBiotech, Inc., Norcross, GA, USA) following the manufacturer's recommendations. The configuration of the antibody arrays is given in Supplementary Figures 1 and 2 of the Supporting Information. Total equivalent amounts of protein (300 μg) from the cell lysates described in the previous section were incubated with the arrays for 2 h at room temperature. Detection was performed with biotin-conjugated antibodies raised against the particular molecules and horseradish peroxidase-conjugated streptavidin. Arrays were visualized using a CCD camera coupled to a Chemidoc 881XRS equipment (Bio-Rad Laboratories). Nonsaturated spots were scanned and converted to densitometric units using the software ScanAlyze.[18] The proteins were represented in duplicate per array. Negative controls and blank spots were used to determine the background. Raw data (intensity value of each spot) were subtracted from the averaged background and normalized according to positive control densities. Differences in protein expression between experimental groups are expressed as fold-change. Proteins showing changes in expression ≥1.3-fold or <1.3-fold were considered to be up-regulated or down-regulated, respectively (because modest changes in expression may have biological significance). Triplicate (IL-1β-treated cells) or duplicate experiments (TNF-α-treated cells) were performed for each treatment. Data are presented as the mean value ± SD.

Statistical Analyses. Results are presented as mean values ± SD (displayed as error bars). When indicated, data were statistically analyzed using PASW statistics 18.0 (SPSS Inc., Chicago, IL, USA), and differences between experimental groups were made using two-tailed unpaired Student's *t* test. Results with a *P* value of <0.001, <0.01, or <0.05 were considered to be significant. Results showing a trend with *P* values <0.1 are also indicated.

RESULTS

Effects of the Urolithins and (or) EA on PGE_2 Levels. Our results showed that exposure to IL-1β (1 ng/mL, 18 h) caused a very significant induction (36.0 ± 1.6-fold; $P < 0.001$) in the production of PGE_2 by the colon fibroblasts, which was completely attenuated by cotreatment with the MIX metabolites (40 μM Uro-A, 5 μM Uro-B, 1 μM EA) ($P < 0.001$) or Uro-A (40 μM) (Figure 1a). Uro-B (5 μM) also reduced the levels of PGE_2 in a significant manner (66.5% reduction, $P < 0.001$), whereas EA had no effect on the synthesis of PGE_2 (Figure 1a). Treatment of the colon fibroblasts with TNF-α, alone or in combination with the metabolites (Figure 1b), or exposure of the cells to the metabolites in the absence of cytokine had no significant effects on the levels of PGE_2.

Effect of the Urolithins and (or) EA on Colon Fibroblast Migration, Cell Viability, and Related Molecular Markers. To investigate whether the mixture of metabolites or the individual compounds exerted any anti-inflammatory phenotypic responses, we next examined the effects of these compounds on the fibroblasts' ability to migrate. The effect of the urolithins and (or) EA, alone or in combination with the cytokines, on the migration of CCD18-Co cells is shown in Figure 2. Induction of migration with IL-1β was moderate (24%, $P < 0.1$) (Figure 2a), and both the MIX metabolites and Uro-A as well as EA significantly inhibited IL-1β-induced migration by 69% ($P < 0.001$), 57% ($P < 0.01$), and 38% ($P < 0.001$), respectively. Colon fibroblast migration was also moderately but significantly induced after treatment with TNF-α for 48 h (29% induction; $P < 0.01$; Figure 2b) and inhibited by cotreatment with the MIX (70%), Uro-A (54%), and EA (19%) ($P < 0.001$). In the absence of the inflammatory cytokines, colon fibroblast migration was significantly inhibited by the MIX metabolites (64%, $P < 0.01$; Figure 2c). Uro-A

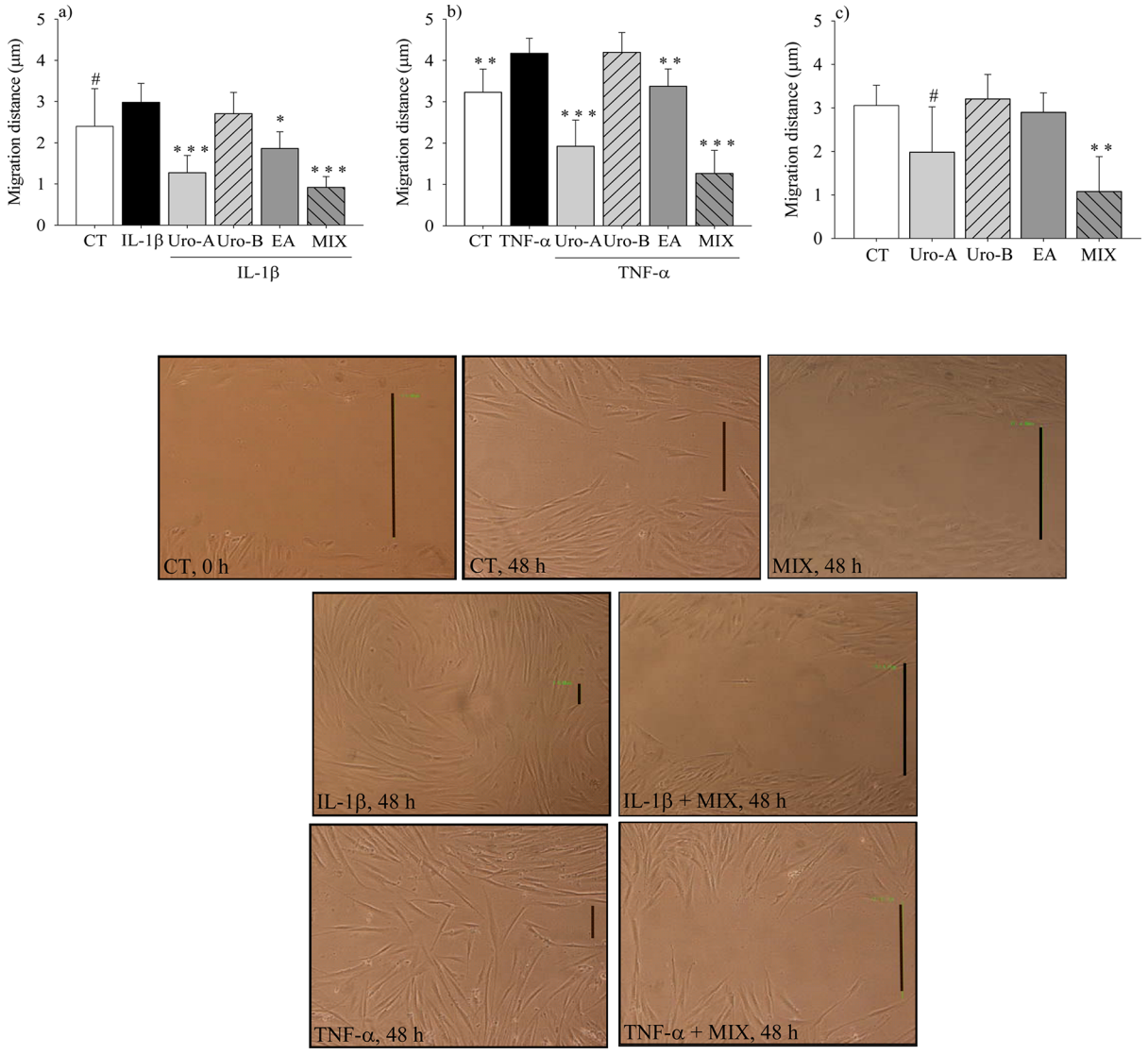

Figure 2. Effects of Uro-A (40 μM), Uro-B (5 μM), EA (1 μM), and a mixture of these molecules (40 μM Uro-A, 5 μM Uro-B, and 1 μM EA; MIX) on CCD18-Co colon fibroblast migration: (a) cotreatment with IL-1β (1 ng/mL); (b) cotreatment with TNF-α (50 ng/mL); (c) in the absence of the inflammatory cytokines. Histograms show the final migrated distance calculated as the difference between the gap distance at time 0 and after 48 h of exposure. Data are representative of three separate experiments (mean value ± SD). Symbols indicate differences from (a, b) cytokine-treated samples and (c) control (CT) cells: #, $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (d) Representative photomicrographs showing migration of CCD18-Co fibroblasts at 48 h in (i) control medium (CT) (panels a, b, or c, CT); (ii) effect of the MIX on resting cells (panel c, MIX); (iii) effect of IL-1β alone (panel a, IL-1 β) or TNF-α alone (panel b, TNF- α); (iv) effect of IL-1β or TNF-α in combination with the MIX (panels a and b, MIX).

exhibited a smaller and less significant inhibitory effect (35%; $P < 0.1$), whereas Uro-B or EA did not have a direct effect on fibroblast migration.

To determine whether exposure of the colon fibroblasts to the urolithins and (or) EA for 48 h was inducing some kind of toxicity (or) alteration of the cell proliferation rate, the effects of the metabolites on total mitochondrial activity were assessed using the MTT assay (Figure 3). Activation with IL-1β alone or in combination with the metabolites did not have a significant effect on the cells viability (Figure 3a). Exposure of the fibroblasts to TNF-α had a small but significant detrimental effect on the cells' mitochondrial activity (35% inhibition, $P < 0.001$; Figure 3b), which was slightly recovered following exposure to the MIX metabolites or Uro-A. Neither the MIX nor the individual metabolites, in the absence of cytokine, caused significant changes in the rates of MTT reduction at the concentrations tested.

We also investigated the changes in the levels of PAI-1 using an ELISA (Figure 4), which was highly up-regulated after treatment with the cytokines (6.5 ± 0.7 and 4.5 ± 0.3 for IL-1β and TNF-α, respectively; $P < 0.001$) and down-regulated following exposure to the MIX or Uro-A for 48 h, more significantly in the IL-1β-treated cells (0.5 ± 0.2, $P < 0.01$, and 0.6 ± 0.3, respectively; $P < 0.05$). In addition, we were not able to detect any significant effects on the levels of PDGF-BB or PDGF-R-β in colon fibroblasts after IL-1β or TNF-α stimulation for 48 h, either alone or in combination with Uro-A or the MIX using the ELISA technique (results not shown).

Search for Further Growth and Migration Factors Modulated by the Cytokines and the ET Metabolites Using Antibody Arrays. Because inhibition of fibroblast

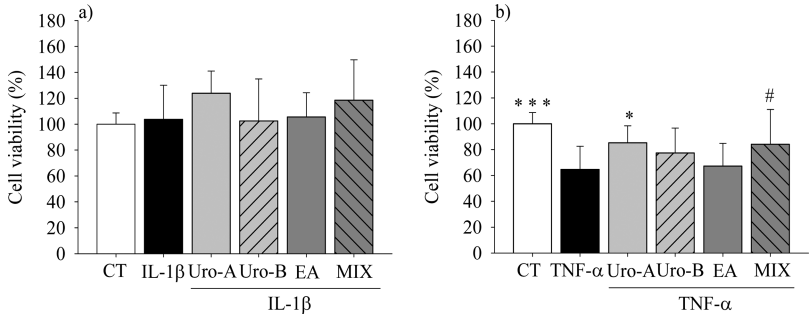

Figure 3. Effects of Uro-A (40 μM), Uro-B (5 μM), EA (1 μM), and a mixture of these molecules (40 μM Uro-A, 5 μM Uro-B, and 1 μM EA; MIX) on CCD18-Co colon fibroblast viability using the MTT assay after 48 h of exposure: (a) cotreatment with IL-1β (1 ng/mL); (b) cotreatment with TNF-α (50 ng/mL). The results from three separate experiments are expressed as mean percentage of untreated control ± SD. Symbols indicate differences from TNF-α-treated samples: #, $P < 0.1$; ∗, $P < 0.05$; ∗∗∗, $P < 0.001$.

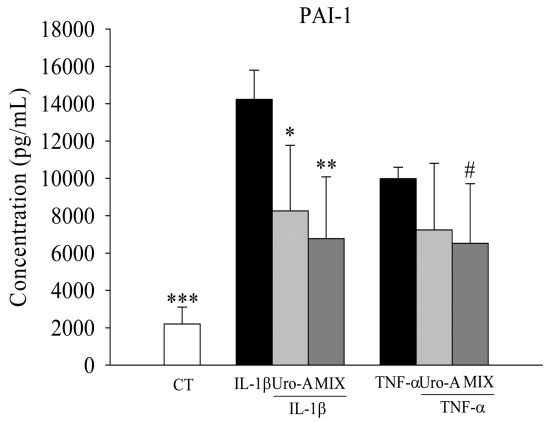

Figure 4. Levels of PAI-1 released to the CCD18-Co colon fibroblast culture media after 48 h of treatment as measured by ELISAs after exposure to IL-1β (1 ng/mL) or TNF-α (50 ng/mL), alone or in combination with Uro-A (40 μM) or the MIX metabolites (40 μM Uro-A, 5 μM Uro-B, and 1 μM EA). Data are presented as the mean value from three independent experiments ± SD. Symbols indicate differences from cytokine-treated samples: #, $P < 0.1$; ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$.

migration was most significant following exposure to the MIX of metabolites and Uro-A, we selected these treatments for the subsequent screening of further changes in growth factors that may be associated with the responses of the CCD18-Co using human antibody arrays. The complete profile of growth factors represented on the array (ranked in order of spot intensity from highest to lowest value) and the differences detected for each between the experimental groups (fold-change) are included in Supplementary Tables 1 and 2 of the Supporting Information. Both cytokines, Il-1β and TNF-α, caused a general up-regulation of many of the growth factors represented on the chip (80 and 60%, respectively), whereas the MIX and Uro-A down-regulated some of those. Fold-changes were also, in general, very moderate, with most changes ranging between 1.3 and 3.0. Table 1 summarizes the comparative results between IL-1β- and TNF-α-treated cells for some of the major growth factor families that participate in cell migration and wound healing. Whereas treatment with IL-1β was concomitant with the up-regulation of all the main members of these growth factors families, exposure to TNF-α did not modify the levels of the TGFβ family, some VEGF members, or some of the macrophage colony-stimulating factors. In the IL-1β-stimulated cells, exposure to the MIX or Uro-A was associated with the down-regulation of most of the growth factors selected, whereas in the TNF-α-treated fibroblasts, results exhibited more variation with some growth factors resulting further up-regulated by exposure to the MIX (FGF6, TGFβ members, some VEGF members, and M-CSF) or Uro-A (bFGF and IGFBP-3). It should be noted that, unlike the results obtained with the ELISAs, the antibody arrays indicated that stimulation of the colon CCD-18Co fibroblasts with each of the cytokines was accompanied by an induction of the levels of PDGF-BB or PDGF-R-β, which were down-regulated by the investigated metabolites of ellagitannins.

Effect of the Urolithins and (or) EA on Monocyte Adhesion to Colon Fibroblasts and Related Molecular Markers. We next investigated the effects of the ET metabolites on THP-1 monocyte adhesion. Incubation of the CCD18-Co cells with IL-1β (1 ng/mL; Figure 5a) or TNF-α (50 ng/mL; Figure 5b) for 12 h significantly increased the monocyte adhesiveness by 50 and 32%, respectively ($P < 0.001$). Of the metabolites tested, Uro-A and EA significantly inhibited monocyte adhesion on IL-1β-treated cells by 20% ($P < 0.05$), whereas Uro-B and EA moderately but significantly inhibited monocyte adhesion on TNF-α-treated cells by 22% ($P < 0.05$). The most significant inhibition was observed following exposure of the cytokine-activated colon cells to the MIX of metabolites (35 and 50% inhibition, respectively, $P < 0.001$). None of the compounds tested had any effect on monocyte adhesion in the absence of the inflammatory cytokines.

We also measured the concentration of selected chemokines and adhesion molecules in control cells and cells exposed to Uro-A or the MIX metabolites (Figures 6) using ELISAs. The results show that stimulation of the CCD18-Co fibroblasts with the pro-inflammatory cytokines IL-1β (1 ng/mL) or TNF-α (50 ng/mL) for 12 h significantly ($P < 0.001$) up-regulated the expression of IL-8 (fold-change = 17.4 ± 3.5 and 12.0 ± 1.6, respectively), CCL2 (2.4 ± 0.2 and 1.8 ± 0.1), ICAM-1 (11.9 ± 0.9 and 8.4 ± 0.6), and VCAM-1 (3.4 ± 1.1 and 3.8 ± 1.1). Cotreatment of the inflamed colon fibroblasts with the ET metabolites revealed that the levels of IL-8 released to the cell culture media were only significantly down-regulated by the MIX (0.6 ± 0.3) and by Uro-A (0.7 ± 0.2) ($P < 0.05$) in the TNF-α-treated cells (Figure 6a). Neither Uro-A nor the MIX metabolites showed any effect on the levels of CCL2 (Figure 6b). However, the MIX metabolites were able to reduce the expression levels of ICAM-1 and VCAM-1 in the IL-1β-treated cells (0.7 ± 0.2, $P < 0.05$) and (0.7 ± 0.1, $P < 0.1$)), respectively (Figure 6c,d). The levels of these two adhesion proteins were shown to be unmodified following treatment of cells with TNF-α and the metabolites (Figure 6c,d). IL-6 was

Table 1. Selected Human Growth Factors Involved in Cell Migration and Wound Healing with Altered Levels of Expression in Human Colon Fibroblasts CCD18Co As Determined by RayBio Antibody Arrays: IL-1β-Treated Cells (IL-1β, 1 ng/mL) vs Control Cells (CT); IL-1β + MIX (40 μM Uro-A, 5 μM Uro-B, and 1 μM EA) Treated Cells (IL-1β + MIX) vs IL-1β-Treated Cells; IL-1β + Uro-A (40 μM) Treated Cells (IL-1β + Uro-A) vs IL-1β-Treated Cells; TNF-α-Treated Cells (TNF-α, 50 ng/mL) vs Control Cells (CT); TNF-α + MIX-Treated Cells (TNF-α + MIX) vs TNF-α-Treated Cells; and TNF-α + Uro-A-Treated Cells (TNF-α + Uro-A) vs TNF-α-Treated Cells[a]

protein name	symbol	Il-1β/CT	Il-1β + MIX/Il-1β	Il-1β + Uro-A/Il-1β	TNF-α/CT	TNF-α + MIX/TNF-α	TNF-α + Uro-A/TNF-α
epidermal growth factor (EGF) family							
epidermal growth factor	EGF	**1.53 (*0.038*)**	**−1.66**	NC	**1.66 (*0.041*)**	**−1.51 (*0.029*)**	−1.42
epidermal growth factor receptor	EGFR	**1.80**	NC	**NC**	**1.99 (*0.093*)**	−2.10 (*0.026*)	**NC**
fibroblast growth factor (FGF) family							
basic fibroblast growth factor	bFGF	**2.78**	**−2.68**	−2.27	**1.32**	**−1.51**	1.81
fibroblast growth factor 4	FGF4	**1.49**	**−1.43**	NC	**1.62**	**−1.53 (*0.001*)**	−1.47
fibroblast growth factor 6	FGF6	**2.13 (*0.021*)**	−2.56 (*0.049*)	−2.28 (*0.054*)	**1.57**	5.68 (*0.007*)	NC
fibroblast growth factor 7	FGF7	**2.94 (*0.014*)**	**−2.21**	−2.01	**1.48**	**−1.96 (*0.008*)**	NC
transforming growth factor β (TGFβ) family							
transforming growth factor β-1	TGFβ1	6.22 (*0.093*)	−1.47	**−2.05 (*0.073*)**	NC	1.48	**−7.19 (*0.044*)**
transforming growth factor β-2	TGFβ2	1.80 (*0.016*)	NC	**−1.59 (*0.001*)**	NC	1.47	−1.53
transforming growth factor β-3	TGFβ3	2.12 (*0.001*)	NC	**−1.69 (*0.001*)**	NC	1.67	−2.45
platelet-derived growth factor (PDGF) family							
platelet-derived growth factor A chain	PDGF-AA	1.96 (*0.044*)	−1.46 (*0.052*)	**−1.37 (*0.067*)**	NC	NC	**−1.55 (*0.068*)**
platelet-derived growth factor subunit B (homodimer)	PDGF-BB	**2.05 (*0.028*)**	**−1.77 (*0.088*)**	**−1.64**	**1.41**	**−1.84**	**−1.55**
platelet-derived growth factor subunit B (heterodimer)	PDGF-AB	**1.88 (*0.032*)**	**−1.51**	**−1.53 (*0.074*)**	**1.33**	**−1.45**	**−1.86 (*0.047*)**
platelet-derived growth factor receptor, α polypeptide	PDGF-R-α	**2.75 (*0.001*)**	**−1.49 (*0.042*)**	**−1.76 (*0.010*)**	**1.53**	**−1.41**	**−2.29 (*0.007*)**
β-type platelet-derived growth factor receptor	PDGF-R-β	**1.78 (*0.091*)**	**−1.56**	NC	**1.51**	**−1.36**	−2.21 (*0.061*)
vascular endothelial growth factor (VEGF) family							
vascular endothelial growth factor A	VEGF	2.66 (*0.001*)	NC	−1.69 (*0.001*)	NC	1.43	NC
vascular endothelial growth factor receptor 2	VEGF R2	2.40 (*0.028*)	NC	**−1.75 (*0.065*)**	NC	1.41	**−1.47**
vascular endothelial growth factor receptor 3	VEGF R3	2.25 (*0.007*)	**NC**	−2.04 (*0.003*)	NC	**NC**	NC
vascular endothelial growth factor D	VEGF-D	2.53 (*0.001*)	−1.39 (*0.040*)	**−1.99 (*0.001*)**	NC	NC	**−1.81**
placental growth factor	PIGF	**2.42 (*0.006*)**	**−1.61 (0.082)**	**−1.66 (*0.031*)**	**1.84 (*0.095*)**	**−2.18 (*0.068*)**	**−2.24 (*0.025*)**
macrophage colony-stimulating factor family							
macrophage colony-stimulating factor 1	M-CSF	**1.77 (*0.028*)**	−1.60 (*0.007*)	**−1.46 (*0.026*)**	**1.52**	5.08	**−2.66 (*0.016*)**
macrophage colony stimulating factor receptor	M-CSFR	1.68 (*0.007*)	**NC**	**−1.53 (*0.041*)**	NC	**NC**	**−1.73 (*0.072*)**
granulocyte-macrophage colony-stimulating factor	GM-CSF	8.05 (*0.029*)	−1.80	−2.22 (*0.093*)	ND	ND	ND
IGF family							
insulin-like growth factor I	IGF-I	**1.92 (*0.021*)**	**−1.51**	**−1.41**	**1.62 (*0.066*)**	**−2.13 (*0.006*)**	**−1.37**
insulin-like growth factor-binding protein 2	IGFBP-2	**1.45 (*0.082*)**	**−1.32**	−1.43 (*0.074*)	**1.40**	**−1.35**	NC
insulin-like growth factor binding protein 3	IGFBP-3	**2.45 (*0.037*)**	**−1.43**	NC	**1.71**	**−1.94 (*0.005*)**	1.40
insulin-like growth factor 1 soluble receptor	IGF-I SR	**1.71**	**−1.51 (*0.057*)**	−1.38	**1.76 (*0.073*)**	**−2.35 (*0.003*)**	NC

[a] Data (fold-change after 48 h of exposure) are the mean value from three (IL-1β-treated cells) or two (TNF-α-treated cells) independent experiments. Cell extracts from two plates were pooled and hybridized onto one antibody array per experiment. Proteins are represented in duplicate on the chip. Cut-off value: up-regulation fold-change ≥1.3, down-regulation fold-change < −1.3. Estimated *P* values <0.1 are indicated. NC, not changed. ND, not detected. Boldface entries indicate comparable results (same change direction) between IL-1β- and TNF-α-stimulated cells. *P* values are given in parentheses.

also highly induced both by TNF-α and IL-1β following 12 and 48 h of incubation to each of the cytokines, but none of the tested metabolites had any effect on the expression on this cytokine (results not shown).

Search for Further Adhesion Proteins Modulated by the Cytokines and the ET Metabolites Using Antibody Arrays. We were also able to search for additional adhesion markers that may be modulated in the CCD-18Co cells following exposure to TNF-α and to TNF-α + MIX metabolites and that may be associated with the regulation of monocyte adhesion to these colon fibroblasts. The complete profile of adhesion proteins represented on the array (ranked in order of spot intensity from highest to lowest value) and the differences (fold-change) detected for each between the experimental groups are included in Supplementary Table 3 of the Supporting Information. A summary of those adhesion markers

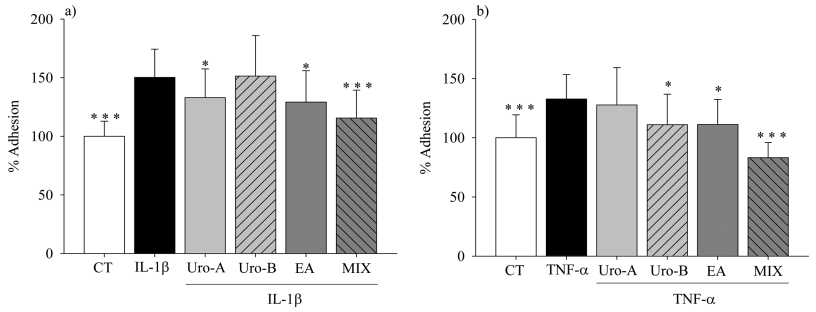

Figure 5. Effects of Uro-A (40 μM), Uro-B (5 μM), EA (1 μM), and a mixture of these molecules (40 μM Uro-A, 5 μM Uro-B, and 1 μM EA; MIX) on adhesiveness of CCD18-Co to THP-1 monocytes: (a) cotreatment with IL-1β (1 ng/mL); (b) cotreatment with TNF-α (50 ng/mL). Colon fibroblasts were exposed to the different treatments for 12 h, and adhesion to monocytes was measured. The results from three separate experiments are expressed as mean percentage of untreated control ± SD. Symbols indicate differences from cytokine-treated samples: *, $P < 0.05$; ***, $P < 0.001$.

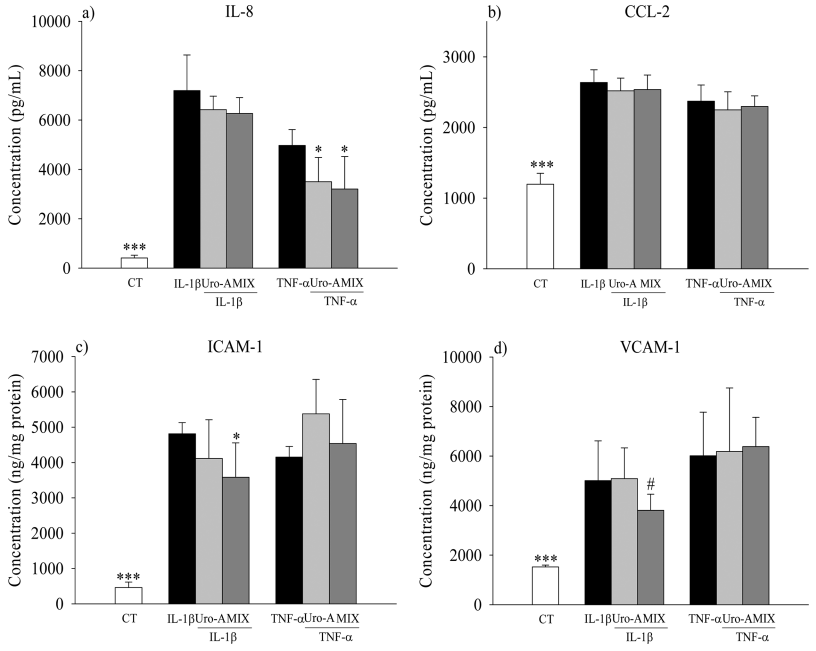

Figure 6. Levels of selected cytokines and adhesion molecules as measured by ELISAs in the supernatants or cell extracts from CCD18-Co colon fibroblasts exposed to IL-1β (1 ng/mL) or TNF-α (50 ng/mL), alone or in combination with Uro-A (40 μM) or the MIX metabolites (40 μM Uro-A, 5 μM Uro-B, and 1 μM EA): (a, b) levels of IL8 and CCL2 released to the cell culture media after 12 h of treatment; (c, d) expression levels of ICAM-1 and VCAM-1 in cell extracts after 12 h of treatment. Cell extracts from two plates were pooled per experiment. Data are presented as the mean value from three independent experiments ± SD. Symbols indicate differences from cytokine-treated samples: #, $P < 0.1$; *, $P < 0.05$; ***, $P < 0.001$.

that were up-regulated by the cytokine and down-regulated by the MIX metabolites is presented in Table 2. In agreement with the ELISAs, densitometric analysis of the arrays showed that IL-8, CCL2, ICAM-1, and VCAM-1 were all highly up-regulated in the CCD18-Co cells following treatment with TNF-α. Cotreatment with the MIX metabolites showed a tendency to down-regulate the levels of IL-8, whereas the levels of ICAM-1 and VCAM-1 were not affected, as also shown by the ELISAs. However, and unlike the results of the ELISAs, the arrays also indicated a small (but significant) down-regulation of the levels of CCL2 by the MIX in the TNF-α-treated cells. Other adhesion proteins such as ALCAM, BCAM, and CDH1 were also found to be up-regulated by TNF-α and down-regulated by the MIX.

DISCUSSION

The current treatment of intestinal chronic inflammation consists of long-term anti-inflammatory therapy that does not exclude relapses, side effects, and surgery. Many dietary polyphenols exert anti-inflammatory effects in animal models and cultured cells and have been proposed as food-derived bioactives to prevent or treat chronic inflammatory diseases. The anti-inflammatory properties of polyphenols may be especially relevant in the intestine, where they can reach considerable concentrations through the regular intake of polyphenol-rich foods.[6] Yet, to fully understand the intestinal anti-inflammatory actions of dietary polyphenols it is crucial to (i) identify and quantify the actual metabolites found in vivo in the gut and (ii) unravel the underlying molecular mechanisms triggered by these metabolites in the specific target cells involved in the intestine inflammatory response. In the present work, we have investigated some of the anti-inflammatory effects and potential molecular mechanisms of the main colon microbiota metabolites derived from the dietary polyphenol ETs against fibroblasts of the colon. We found that a mixture of Uro-A, Uro-B, and EA, at concentrations representative of those that may be achieved in the gut through the diet,[9] significantly inhibited two critical processes of the intestinal

Table 2. Selected Adhesion Proteins with Altered Levels of Expression in Human Colon Fibroblast CCD18Co As Determined by RayBio Antibody Arrays: TNF-α-Treated Cells (TNF-α, 50 ng/mL) vs Control Cells (CT) and TNF-α + MIX (40 μM Uro-A, 5 μM Uro-B and 1 μM EA) Treated Cells (TNF-α + MIX) vs TNF-α-Treated Cells[a]

protein name	symbol	TNF-α/CT	TNF-α + MIX/TNF-α
interleukin 8	IL-8	7.02 (*0.017*)	−1.75
chemokine (C−C motif) ligand 2	CCL2	2.98 (*0.037*)	−1.41 (*0.003*)
intercellular adhesion molecule 1	ICAM-1	1.53	NC
vascular cell adhesion molecule 1	VCAM-1	3.92 (*0.001*)	NC
activated leukocyte cell adhesion molecule	ALCAM	1.72	−1.61
basal cell adhesion molecule (Lutheran blood group)	BCAM	1.44	−1.69
cadherin 1, type 1, E-cadherin (epithelial)	CDH1	1.85 (*0.015*)	−2.70 (*0.044*)

[a] Data (fold-change after 12 h of exposure) are the mean value from two independent experiments. Cell extracts from two plates were pooled and hybridized onto one antibody array per experiment. Cutoff value: up-regulation fold-change ≥1.3, down-regulation fold-change < −1.3. Proteins are represented in duplicate on the chip. Estimated *P* values < 0.1 are indicated in parentheses. NC, not changed.

inflammatory response: fibroblast migration and monocyte adhesion. These effects were associated with a significant down-regulation of the levels of PGE_2, PAI-1, and IL-8, key regulators of cell migration and adhesion. Of the three metabolites tested, Uro-A exhibited the most significant anti-inflammatory effects.

In this study we investigated the response of the myofibroblast-like human cell line (CCD-18Co) because these cells are noncancerous and are representative of colon fibroblasts that lie underneath the gut epithelium. These cells constitute an important and active component in the maintenance of the intestinal mucosa. Although colon fibroblasts are less likely to be in direct contact with the luminal dietary compounds, transient increases in the permeability of the tight junctions under inflammatory conditions make possible the passage of many nutrients and small molecules through the paracellular route.[19] In addition, chronic inflammation may lead to disruption of the epithelial barrier, allowing for direct contact of luminal content with the cells resident in the lamina propria. Therefore, it is conceivable that colon fibroblasts may become exposed to significant quantities of colon dietary metabolites. Uro-A was the most abundant ET-derived metabolite detected in the feces of pigs and humans, followed by Uro-B and trace quantities of EA.[8,20] In rats fed a high dose of pomegranate (216 mg/kg; human equivalent dose = 2.4 g per a 70 kg person), Uro-A (7−34 μM), Uro-B (2−65 μM), and EA (minor quantities) were quantified in the colon.[9] On the basis of these results as well as on the maximum solubility of urolithins and EA in the cell culture medium (40 and 30 μM, respectively),[10] we treated the colon fibroblasts with 40 μM Uro-A, 5 μM Uro-B, and 1 μM EA.

Myofibroblasts in the colon mucosa play an important modulatory role in the intestinal inflammatory response partially by releasing PGE_2.[21] We had previously established the association between the consumption of PE or Uro-A with the down-regulation of the levels of PGE_2 in the rat colon mucosa under inflammatory conditions.[7] Using human colon CCD-18Co fibroblasts, we had also evidenced that Uro-A and Uro-B, at 1 and 10 μM concentrations, were able to counteract the induction of PGE_2 by the pro-inflammatory cytokine IL-1β,[10] but a synergistic effect of the ET metabolites was not investigated. In the present work, we have confirmed that a mixture of the two urolithins and EA, at concentrations representative of those that may be found in the gut through the consumption of ET-rich products, is able to fully reverse the IL-1β induction of PGE_2 and that these effects are mainly due to Uro-A. However, TNF-α, another key inflammatory cytokine, or the metabolites themselves did not show any effect on the levels of PGE_2. Although TNF-α has been reported to up-regulate the levels of this prostaglandin in CCD-18Co cells, the degree of inducibility varies considerably, and it has been shown to be much more pronounced with IL-1β than with TNF-α.[15] More in agreement with our results, in a recent study, it was shown that exposure of the CCD-18Co cells to TNF-α (8.3 ng/mL, 4 h) did not have a significant effect on the production and secretion of PGE_2 by these cells.[22] The prostaglandin PGE_2 has an important regulatory role on several key fibroblast functions including fibroblast migration and has been reported to be able to stimulate or inhibit fibroblast migration.[21,23] An important mechanism involved in wound healing and tissue formation in the intestine is the migration of colonic lamina propria fibroblasts from neighboring tissue to inflammation site and damaged mucosa. This response is considered to be mediated by a complex chemotactic gradient, with some molecules inducing or enhancing migration and others reducing the ability of these cells to migrate. In addition to PGE_2, the pro-inflammatory cytokines, TNF-α and IL-1β, have also important regulatory effects on cell migration.[12−14] To further explore the response of the colon fibroblasts to exposure to the ET metabolites, we investigated the effects of these compounds, alone or in the presence of the pro-inflammatory cytokines, on the ability of these cells to migrate. Under the conditions of our assay, both TNF-α and IL-1β were able to increase colon fibroblast migration, in agreement with a pro-fibrotic effect important to recruit activated fibroblasts into wound sites as part of the inflammatory and healing process. This effect was inhibited by cotreatment with the ET metabolites, suggesting an apparent interference with the normal wound healing and tissue restitution. In contrast to our finding, TNF-α has also been reported to reduce the migration of cultured colon lamina propria fibroblasts.[24] Differences on the effects of the pro-inflammatory cytokines on cell migration, and in particular of TNF-α, can be understood only in view of the complexity of its actions. TNF-α may exert both immunomodulatory and disease-suppressive activities but also initiate and sustain chronic inflammatory conditions.[25] The effects depend on many factors including cell microenvironment and tissue source or the dose and time of exposure.[26] When the homeostatic mechanisms controlling wound healing become disordered in chronic intestinal inflammatory diseases, reduction of the colon fibroblast migration by the ET metabolites might protect against excessive fibrosis.

Another key molecule associated with inflammatory reactions in the intestine mucosa and with a crucial regulatory role on fibroblast migration is PAI-1.[27] Although the net effect of PAI-1 on cell motility depends on many factors (i.e., ECM composition, concentration of PAI-1, expression of cell receptors, etc.), in general, the expression of PAI-1 is highly induced in the migratory phenotype, whereas migration is reduced in low PAI-1 levels.[28] In agreement with this, our results indicate that induction of colon fibroblast migration by TNF-α and IL-1β is associated with a significant induction of PAI-1 and that inhibition of the process by the MIX metabolites or Uro-A is concomitant with the down-regulation of PAI-1. We further

investigated the effects of the ET metabolites on several growth factor families known to be involved in cell growth and migration using antibody arrays. Under pro-inflammatory conditions such as those occurring during acute wounds, the cytokines IL-1β and TNF-α are up-regulated and activate other cells such as fibroblasts to produce high levels of various growth factors.[29,30] Also, transcriptional analyses of migrating human keratinocytes have been associated with the up-regulation of various growth factors, that is, VEGF, PDGF-β, and TGF-β1.[31] In agreement with this, our results show that exposure of the colon fibroblasts to the inflammatory cytokines IL-1β or TNF-α was concomitant with a general up-regulation of some key growth factors, such as members of the EGF, FGF, PDGF, and IGF families. Of particular interest, the PDGF family and its receptors and, more specifically, PDGF-BB- and PDGF-Rβ-induced signaling, are likely important in the development of intestinal fibrosis.[32] Our results using the antibody arrays also show a general down-regulation of these PDGFs by the MIX metabolites or Uro-A, suggesting a potential preventive mechanism of these molecules against fibrotic disorders. Although we were not able to reproduce the changes on the levels of PDGF-BB or PDGF-R-β using some ELISAs, our array results are in good agreement with those previously published by us,[11] in which we also showed that PDGFBB, PDGFAB, PDGFAA, and PDGF-R-β were all up-regulated in human aortic endothelial cells by the inflammatory cytokine TNF-α and down-regulated after exposure to Uro-A. The regulation of the levels of the placental growth factor (PIGF) should also be noted because it has been reported that high levels of PIGF induce the expression of PAI-1[33] and that PIGF absence strongly inhibits mucosal intestinal angiogenesis in acute colitis.[34] Our results show that both the MIX metabolites and Uro-A down-regulated the levels of PIGF in the cytokine-induced colon fibroblasts. A similar response was also seen in human aortic endothelial cells.[11]

During inflammation, chemokines such as IL-8 and CCL-2 as well as adhesion molecules (ICAM-1) are induced in colonic subepithelial myofibroblasts in response to IL-1β and TNF-α.[35,36] These molecules are involved in the processes of infiltration and adhesion of neutrophils and monocytes to the site of inflammation and exhibit high levels of expression in inflammatory colon diseases.[37] To further characterize the anti-inflammatory mechanisms of the ET metabolites, we also examined the effects of these metabolites on the adhesion of monocytes to colon fibroblasts as well as on the production of relevant chemokines and adhesion proteins. Our results show that monocyte adhesion was moderately induced by the pro-inflammatory cytokines and attenuated, primarily, by the MIX metabolites. This reducing effect was, however, related to apparently different mechanisms in the TNF-α- and IL-1β-treated cells. Like this, reduction of monocyte adhesion in the TNF-α-induced cells by the MIX was concomitant with the down-regulation of IL-8 secretion. This down-regulation was also observed following treatment with Uro-A, in agreement with previous results showing that Uro-A diminished the levels of IL-8 in TNF-α-stimulated human endothelial aortic cells.[11] In the IL-1β-treated cells, the MIX did not affect the levels of IL-8 but caused a small decrease in the adhesion molecules ICAM-1 and VCAM-1. A similar response in which the reduction of ICAM-1 expression was observed in cells exposed to IL-1β but not in those treated with TNF-α has been reported as specific effects of anti-inflammatory corticosteroids on Crohn's fibroblasts.[36] In contrast to this, other polyphenols such as apple polyphenols have been shown to inhibit sICAM-1 and down-regulate IL-8 protein expression in a colon carcinoma cell line stimulated with both TNF-α and IL-1β.[38] With regard to CCL2 regulation, results were less clear because we were able to detect only a small down-regulation of this monocyte chemoattractant protein following treatment with the MIX metabolites in the TNF-α-induced fibroblasts using antibody arrays.

Our results show some agreement with but also some differences from the results obtained by RayBio antibody arrays and those obtained with the ELISAs. Although the antibody array technology has improved substantially and has been efficiently validated by ELISAs,[39,40] it is still a rather expensive technique, which limits the number of samples that can be done and the reproducibility of the data. For proteins with higher levels of expression and (or) exhibiting greater changes such as those induced by the cytokines (IL-1β or TNF-α), results were more easily replicated and confirmed by the ELISAs. The concentrations detected with the ELISAs were in the range of ng/mL to pg/mL with changes from ~500 to 7000 pg/mL for IL-8 and from ~1000 to 2500 pg/mL for CCl2 in IL-1β-treated cells. The antibody arrays detected some of the same molecules and changes, which show they have a high sensitivity. There are not many published studies reporting cytokine levels in CCD18Co cells under the same inflammatory conditions used by us. Lippert et al. measured the concentrations of IL-8 in CCD18Co cells (5−50 pg/μg protein) and in isolated colon fibroblasts (10−180 pg/μg protein) in specific inducing culture media.[41] The levels of CCL2 in cancer-associated fibroblasts can vary from 350−560 to 2950−1800 pg/mL after exposure to TNF-α.[42] For modest changes such as those induced by dietary compounds at low concentrations, results are less reproducible. We cannot disregard the possibility that some of the discordances between the arrays and the ELISAs may also be caused by differences between the antibodies used in each technique.

In conclusion, there are an increasing number of in vitro studies in the literature looking at the bioactivity of polyphenols at conditions more representative of the in vivo situation following the dietary intake of these compounds, that is, testing low concentrations of the appropriate mixed metabolites against suitable cell models. It does appear that, in general, under these conditions the metabolites induce very modest phenotypic responses associated also with a very modest modulation of multiple target proteins (as may be expected for dietary compounds). These effects are indeed more complex to validate than those resulting from the use of pharmacological doses of a particular drug or compound and will demand further efforts to provide evidence of the role of polyphenol metabolites in human health effects. The work presented here is a step forward in this direction. Overall, our results support the hypothesis that micromolar concentrations of polyphenol-derived metabolites that can be achieved in the gut through the diet can exert immunomodulatory effects on cells of the intestinal barrier and contribute to the prevention of intestinal inflammatory diseases.

■ ASSOCIATED CONTENT

Supporting Information

The configuration of the RayBio antibody arrays is given in Supplementary Figures 1 and 2. The complete profile of growth factors and adhesion proteins represented on the array (ranked in order of spot intensity from highest to lowest value) and the

differences detected for each between the experimental groups (fold-change) are included in Supplementary Tables 1−3. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Phone: +34-968-39-6276. Fax: +34-968-39-6213. E-mail: mtconesa@cebas.csic.es.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

IBDs, inflammatory bowel diseases; CD, Crohn's disease; UC, ulcerative disease; PE, pomegranate extract; EA, ellagic acid; ETs, ellagitannins; CCD-18Co, human colon fibroblasts; PBS, phosphate-buffered saline; IL-1β, interleukin one beta; TNF-α, tumor necrosis factor alpha; Uro-A, urolithin A; Uro-B, urolithin B.

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