Inhibition of proliferation, migration, and adhesion of skin fibroblasts by enzymatic poly(gallic acid) grafted with L-Arginine

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ABSTRACT

Psoriasis and atopic dermatitis (AD) are characterized by enhanced skin inflammation, which results in hyper-proliferation and the recruitment of immune cells into the skin. For that reason, it is needed a chemical capable to reduce cell proliferation and the recruitment of cells. The search for new molecules for therapeutic skin treatments mainly focuses on the antioxidant and anti-inflammatory properties, highlighting the rheological properties of polymeric polypeptides. We studied L-arginine (L-Arg) grafted (-g-) to enzymatic poly(gallic acid) (PGAL). The latter is a multiradical antioxidant with greater properties and thermal stability. The derivative was enzymatically polymerized in an innocuous procedure. The poly(gallic acid)-g-L-Arg molecule (PGAL-g-L-Arg) inhibits bacterial strains which also have been involved in the progression of psoriasis and AD. However, it is important to analyze their biological effect on skin cells. The cell viability was analyzed by calcein/ethidium homodimer assays and crystal violet. The proliferation and cell attachment were determined by a curve of time and quantitation of the optical density of crystal violet. To analyze the cell migration a wound-healing assay was performed. This synthesis demonstrates that it is not cytotoxic at high concentrations (250 μg/mL). We observed a decrease in the proliferation, migration, and adhesion of dermal fibroblasts in vitro but the compound could not avoid the increase of reactive oxygen species in the cell. Based on our findings, PGAL-g-L-Arg is a promising candidate for treating skin diseases such as psoriasis and AD where decreasing the proliferation and cell migration could help to avoid inflammation.

Introduction

The design of new molecules capable of modifying cell properties is challenging because their synthesis must accomplish specific characteristics within biological frameworks. For example, psoriasis and atopic dermatitis (AD) are two diseases characterized by hyperproliferation in the skin cells due to a high inflammation process (1, 2). The causes of both diseases could be genetic, environmental, and immunological. The final phenotype of these pathologies in the skin is exacerbated inflammation, resulting in the formation of plaques with the high proliferation of skin and immune cells, intense itch, eczematous lesions, and discomfort (3–5). Even when the molecular mechanisms that induce the lesions in both diseases could be different, they have in common the increase of proliferation and the migration of immune cells to the lesion site. In this regard, avoiding these cellular actions could help avoid skin lesions.

It should be noted that bacterial infections add to these lesions, and some therapies are required to inhibit the proliferation of pathogens (6–8). However, therapies mainly focus on decreasing inflammation, where the use of different drugs depends on the severity. The first-line anti-inflammatory therapy is the short-term use of topical corticosteroids. Systemic immunosuppressants (non-biologics) are the second line of treatments for mild to severe disease, and many studies have shown excellent improvements in patient's health; the most common immunosuppressants are methotrexate, ciclosporin, and acitretin.

Alternatively, biological treatments have emerged during the last decade, consisting of human antibodies to tackle specific proteins towards immunosuppressors, immunomodulators, or antiproliferative effects. For pso-
riasis treatment, the most common ones are anti-tumor necrosis factor (anti-TNF) antibodies, whereas anti-interleukin-13 (anti-IL-13) antibodies are prescribed for AD (7, 9–11). However, these treatments are not indicated in all patients, and treatments are usually expensive; besides, some have non-desired side effects on the body. Therefore, the research on non-expensive molecules to decrease the hyperproliferation of cells, inflammation, sepsis, and the migration of immune cells to the site of the lesions is encouraging. These properties have been described for many natural compounds used as adjuvant therapy in several skin treatments by reducing patient’s exposure to aggressive chemicals or irradiation procedures such as phototherapy. Even when many natural compounds (mainly antioxidants) have been tested to decrease inflammation and cell proliferation, one big problem is guaranteeing the quantity and quality of the products obtained from plants because they can be different from region or from season to season, for that reason the synthesis of a new compound in the laboratory allows guarantee the quality of the products. According to previous studies, GA and its enzyme-mediated polyoxidated PGAL could be beneficial in treating psoriasis and AD since they have antioxidant, bactericidal, anti-inflammatory, cardioprotective, and anti-cancer properties. GA can decrease the expression of keratin 16 and keratin 17, which are markers of psoriasis (12); in one study, GA was encapsulated in chitosan nanoparticles, reducing the hyperproliferation of keratinocytes and showed anti-inflammatory properties (13). Polyphenol has been tested in an in vivo AD mouse model in combination with another natural compound and suppressed the release of pro-inflammatory cytokine IL-6, chemokine (C-C motif) ligand 7 (CCL7), and C-X-C motif chemokine ligand 8 (CXCL8) (14).

GA also induces G2/M arrest of the cell cycle in breast cancer cell lines, reducing cell proliferation (15). High solubility in physiological water is essential for many applications, and the low thermal and photo stabilities of GA can be improved by PGAL, an enzyme-mediated multiradical polymer with antioxidant, anti-inflammatory, and cytoprotective properties (16–18). There is also evidence that PGAL can arrest the cell cycle in G2/M phase 2 cancer of colon cell lines (HCT116 and HT29) (18). On the other hand, L-Arg is an amino acid that has been incorporated into different materials for wound healing (19, 20). The beneficial effects of this amino acid for the skin have been tested in combination with tryptophan as antimicrobial peptides, showing bactericidal effects on methicillin-resistant S. aureus isolated from the skin (21) and increasing the elasticity of the treated skin (22). A fish model for wound healing has shown that a diet with L-Arg improves wound closure, decreases the expression of the pro-inflammatory tumor necrosis factor (TNF-α), and increases the expression of the transforming growth factor β1 (TGF-β1), which is related to tissue regeneration (23), these characteristics are desirables for a medicine to treat the lesions in psoriasis and AD. L-Arg is readily grafted onto PGAL (PGAL-g-L-Arg) to improve the biological characteristics with potential anti-bacterial and antioxidant activities as recently demonstrated (24). The anti-bacterial capacity of PGAL-g-L-Arg is also desired in the treatment of psoriasis and AD since the infections could increase the inflammation and the size of the lesions of the skin. This work demonstrates the biological effect on dermal fibroblasts, highlighting the promising potential of PGAL-g-L-Arg as an adjuvant for DA and psoriasis treatments.

Materials and Methods

Production of PGAL-g-Arg

The microwave-assisted grafting of L-Arg onto PGAL, including the synthesis of the latter, was carried out following a previously reported procedure (24). The proton nuclear magnetic resonance (1H-NMR) spectra corroborated the molecular structure in a Varian MR-400 (USA) spectrometer in D2O; Fourier-transform infrared spectroscopy (FTIR) spectra were acquired in a Perkin-Elmer Attenuated Total Reflection (ATR) FTIR Spectrum-400 equipment. Size exclusion chromatography (SEC) was used to assess the average molecular weight following the earlier reported procedure (24), providing a molecular weight in number (Mn) of 7.3 kDa for the PGAL-g-L-Arg sample used in this work.

Cell culture

Fibroblasts were obtained from the foreskin upon written consent (17). Cells were cultured in DMEM/F12 medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Gibco). Trypsin at 0.25% was used (Gibco) for 5 min, and a supplemented medium was added to perform cell passage. The suspension was centrifugated at 1,500 rpm for 5 min, and the obtained cells were counted in a hemocytometer and seeded at the desired concentration.

Cytotoxicity

Cytotoxicity was measured via calcein AM and EthD-1, using live and dead cells for the cytotoxicity kit (Thermo) and following the manufacturer’s instructions. Briefly, 1 μM calcein AM and 2 μM of EthD-1 were diluted in the cell culture medium for 1 hour. Subsequently, the cells were washed twice with PBS 1× (Gibco), and a fresh medium was added. Photographs were taken in an epifluorescence microscope (Zeiss). Additionally, cells were stained with crystal violet (CV) 0.1% (Sigma) to quantify the number of cells attached to the cell culture plates. In a 96-cell culture plate (Corning), 3,000 cells were seeded and incubated with a supplemented medium for 24 h. The treatments were conducted with the following concentrations of PGAL-g-L-Arg: 1, 10, 100, 250, 500, and 1,000 μg/mL; the control group was untreated. The cells were incubated for another 24 h, and subsequently, the Calcein/EthD-1 or crystal violet stain tests were performed. For the stain with CV at 0.1%, cells were washed with PBS 1X and fixed with glutaraldehyde at 1.1% (Sigma) for 10 min; after three washes with distilled water, the cells were stained with CV at 0.1% for 10 min. Cells were washed with water 10 times, and acetic acid at 10% was added. Plates were read in a spectrophotometer (Synergy/HTX) at 570 nm. All experiments were performed in triplicate and three independent assays.

Cell adhesion assay

For this assay, 5,000 cells were seeded in 96-well culture plates (Corning) and grown in supplemented medium to determine whether PGAL-g-L-Arg affects cell adhesion. Cells were washed with PBS 1X and fixed with glu-
Cell proliferation

Five hundred cells were seeded in 96-well culture plates, grown in supplemented medium, and incubated with PGAL-g-L-Arg (250 µg/mL) or without as control to determine cell proliferation. Cells were fixed and stained with CV for 0.25, 0.5, 1, 2, 3, 5, and 7 days, and the plates were read as described previously.

Cell migration

For the cell migration assays, 150,000 cells were grown in 12-well culture dishes (Corning) and incubated in the supplemented medium until confluency. Plates were treated with 10 µg/mL of mitomycin C (Sigma) for 4 h to suppress cell proliferation and subsequently washed, followed by a scratch across the wells in the confluent cells, using a 200-µL micropipette tip. Then, 250 µg/mL of PGAL-g-L-Arg was added to the corresponding plates. Photographs were taken at 0, 24, 48, and 72 h, using an inverted microscope (Zeiss) to evaluate wound closure.

Statistical analysis

All assays were performed in triplicate and three independent experiments. Normality was evaluated using the Shapiro-Wilk test, followed by the t-student’s test; a probability level of p < 0.05 was considered significant.

Results

PGAL-g-L-Arg shows low cytotoxicity

Analyzing the cytotoxicity of the newly synthesized compounds is essential to propose them as possible medicaments; in this work, dermal fibroblasts were used to analyze the effect of the PGAL-g-L-Arg on them. Experimental evidence with human dermal fibroblasts corroborates the lack of cytotoxicity for PGAL-g-L-Arg. For this, a curve of different molecule doses (0, 10, 100, 250, 500, and 1,000 µg/mL) was added to the fibroblast cultures. Although analysis of the cells’ presence and morphology after 24 h showed the same quantity of fibroblasts attached to the cell culture dishes (250 µg/mL), with the typical spiraled fibroblast morphology, it can be observed that the cells treated with 500 and 1000 µg/mL of PGAL-g-L-Arg showed rounded morphology that could indicate detachment of the culture plates (Fig. 1a and b).

In cell viability assays, it should be noted that at high concentrations (500 and 1,000 µg/mL), most of the cells attached to the cell culture plates are viable, but as was shown in Figure 1, round fibroblasts were present, suggesting poor attachment to the cell culture plates. The number of viable cells was confirmed by Calcein AM (AM = ace-toxymethyl) and ethidium homodimer-1 (EthD-1) assays. Almost all cells were viable at 250 µg/mL. Similar behavior was found for cells treated with 500 µg/mL of PGAL-g-L-Arg, although the number of cells in the latter was reduced, confirming that PGAL-g-L-Arg is not cytotoxic but affects cell adhesion (Fig. 2 a, b, and c).

After these first results, it was decided to continue the cellular evaluation of the PGAL-g-L-Arg using only the concentration of 250 µg/mL, which is high but does not affect dermal fibroblasts’ cell viability.

PGAL-g-L-Arg decreases cell attachment

Under PGAL-g-L-Arg treatment, the highest concentration of round fibroblasts was observed after 24 h. This

Figure 1. PGAL-g-L-Arg maintains fibroblast’s phenotype at high concentrations. (a) Representative photographs of fibroblasts treated with different concentrations of PGAL-g-L-Arg and stained with crystal violet. (b) Absorbance was measured for each experimental condition. Scale bars correspond to 200 µm.

Figure 2. Dermal fibroblasts treated with 250 µg/mL of PGAL-g-L-Arg are metabolically active. Human dermal fibroblasts treated with different concentrations of PGAL-g-L-Arg; in green, fibroblasts positive to calcein (viable cells), in red, fibroblasts positive to EthD-1 (dead cells) (a). Numbers of viable and dead cells present in the different experimental conditions (b). Percentages of viable and dead cells in the different experimental conditions. Scale bars correspond to 100 µm (c).

Figure 3. PGAL-g-L-Arg Prevents cell attachment. Cells were attached to cell culture plates and stained with crystal violet at different time points and treated with 250 µg/mL of PGAL-g-L-Arg (a). The number of cells attached in the different periods and experimental conditions (b). Scale bars correspond to 200 µm; error bars represent standard error.
observation could be explained by an effect of the PGAL-g-L-Arg on cell adhesion of the dermal fibroblast; for that reason, cell adhesion was evaluated. There were analyzed at different times to test cell adhesion. The adhesion of cells during 15, 30, 45, 60, and 120 min evidenced that PGAL-g-L-Arg impedes cell attachment at the early stages, and even at 120 min, cell attachment was prevented. The impediment of cell adhesion was significant compared to the control in all the time points, and it was more evident at 120 minutes when the cells started to show an expansion of their cytoplasm in the control group (Fig. 3).

PGAL-g-L-Arg decreases cell proliferation

The effect of PGAL-g-L-Arg on cell proliferation was evaluated for seven days. As observed in the cytotoxicity assays, the morphology of the cells was not changed when treated with PGAL-g-L-Arg compared to the control group. During the first 3 days, there were no differences in the number of cells compared to the control. However, after 5 and 7 days, cell number was significantly reduced in the treatments with PGAL-g-L-Arg. Interestingly, treatment with PGAL-g-L-Arg resulted in clustered cell growth (Fig. 4). This effect could be of great biomedical importance for skin diseases like psoriasis and AD.

PGAL-g-L-Arg decreases cell migration

Another essential process in lesions observed in psoriasis and AD is the migration of immune cells at these tissues, to test the effect of PGAL-g-L-Arg on cell migration, a wound-healing assay was conducted using human dermal fibroblasts. It should be noted that after cell confluence, the cells were treated with mitomycin C to avoid proliferation and only to evaluate cell migration. After the cells were treated with 250 μg/mL PGAL-g-L-Arg, wound closure was monitored until day 3. In the control group, the cells started to migrate within 24 hours, and the gap was filled on the third day, whereas in the group treated with PGAL-g-L-Arg, only a few cells migrate at the scratch zone on the third day, so they could not close the gap (Fig. 5).

Discussion

Research on new molecules that help improve human health is crucial to avoid secondary effects and guarantee the safety of the patients. Different materials have been designed with antioxidant, anti-inflammatory, and bactericidal activities, promoting wound healing (25, 26). However, many of these properties induce the proliferation of cells in the skin, which is not desired for the treatment of some diseases. Diseases such as psoriasis and AD have a similar phenotype but not necessarily the same molecular mechanisms of action, although the same molecule may be capable of avoiding cell hyperproliferation and immune cell infiltration, which causes the typical red patches and scaly skin in both diseases.

Here, we demonstrate that PGAL-g-L-Arg can decrease the proliferation, migration, and adhesion of human dermal fibroblast cells. The specialized literature describes several antioxidants as therapy or adjuvants in treating psoriasis and AD because their antioxidant capacity is related to immunomodulation (27–29). The PGAL-g-L-Arg has an antioxidant capacity in vitro, besides it is clear, that PGAL-g-L-Arg is not cytotoxic for dermal fibroblasts at high concentrations (250 μg/mL) but inhibits the growth of Staphylococcus aureus (24), as reported recently, which is an undesired microorganism involved in skin damage in both psoriasis and AD (30–32). The effect of the PGAL-g-L-Arg in cell proliferation reduction could be caused by the arrest in cell cycle, and this was observed when colon cancer cell lines were treated with PGAL, the cells decrease the percentage of cells in the phases G0-G1 and increase the percentage of the cells in the phase S (18). It would be interesting to analyze the molecular mechanism by which PGAL-g-L-Arg reduces cell proliferation. The complexity of psoriasis and AD involves many different cells, making it essential to analyze each one to determine the effect of synthesized PGAL-g-L-Arg. These properties of PGAL-g-L-Arg point to its potential use for the adjuvant treatment of psoriasis and AD since in these diseases, there is a high proliferation of the cells as a result of hyper inflammation; also, the recruitment of cells is essential to create the inflammation environment, so the PGAL-g-L-Arg could help to avoid the cell migration to avoid the recruitment of the cells in the lesion site. However, further work is underway to prove the immunomodulatory effects and assess these properties in an animal model.

The PGAL-g-L-Arg has the property of decreasing cell proliferation, migration, and adhesion of skin cells in...
vitro. It is proposed as an adjuvant for the treatment of psoriasis and AD.

**Compliance with Ethical Standards**

**Acknowledgments**

RSS has received research grants from Consejo Nacional de Ciencia y Tecnología (CONACYT) number SEP-BASICA FSSEP02-C-2018-2 #A1-S-16109. MGS has received research grants from DGAPA-PAPIIT IN200520. MGT has received research grants from Cátedras CONACYT, project number 376. MAHV has received scholarships in doctoral studies from CONACYT number 299544.

**Conflict of Interest**

The authors declare that they have no conflict of interest.

**Authors’ contributions**

COS, MAPD, MG and RSS contributed to the conception and design, data collection, analysis, and interpretation, and writing and critical revision of the article. VML, NZJ, MGT, GLG and MAHV contributed to data analysis and interpretation as well as the writing and critical revision of the article.

**Ethical approval**

This article does not contain any studies with human participants or animals performed by any of the authors.

Data will be made available on reasonable request.

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