Complement Activation by Pulp Capping Materials Plays a Significant Role in Both Inflammatory and Pulp Stem Cells' Recruitment

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Abstract

Introduction: The role of complement, especially through the C5a fragment, is well-known for the initiation of inflammation. Its involvement in regeneration has been shown more recently by the recruitment of mesenchymal stem cells. C5a can be produced locally by the pulp fibroblasts in response to injury or infection. This work aims to investigate the effect of different pulp capping biomaterials on complement activation and its possible influence on inflammatory and pulp stem cell recruitment. **Methods:** Conditioned media were prepared from 3 pulp capping biomaterials: Biodentine (Septodont, Saint-Maur-des-Fosses, France), TheraCal (BISCO, Lançon De Provence, France), and Xeno III (Dentsply Sirona, Versaille, France). Injured pulp fibroblasts were cultured with these conditioned media to analyze C5a secretion using an enzyme-linked immunosorbent assay. Dental pulp stem cells (DPSCs) were isolated from human third molar explants by magnetic cell sorting with STRO-1 antibodies. The expression of C5a receptor on DPSCs and inflammatory (THP-1) cells was investigated by immunofluorescence. The migration of both DPSCs and THP-1 cells was studied in Boyden chambers. **Results:** Pulp fibroblast production of C5a significantly increased when the cells were incubated with TheraCal- and Xeno III–conditioned media. The recruitment of cells involved in inflammation (THP-1 cells) was significantly reduced by Biodentine- and TheraCal-conditioned media, whereas the migration of DPSCs was reduced with TheraCal- and Xeno III–conditioned media but not with that of Biodentine. The involvement of C5a in cell recruitment is demonstrated with a C5a receptor–specific antagonist (W54011). **Conclusions:** After pulp injury, the pulp capping material affects complement activation and the balance between inflammation and regeneration through a differential recruitment of DPSCs or inflammatory cells. *(J Endod 2017;■:1–7)*

Key Words

Cell migration, complement, dentin-pulp regeneration, pulp capping biomaterials

Significance

The complement is involved in pulp inflammation and regeneration. The balance of these processes is crucial in vital pulp therapy. A better understanding of the recruitment of the cells involved in these events could help in the choice of direct pulp capping materials and the development of new materials with a predictable clinical outcome.

Vital pulp therapy is performed clinically by applying a pulp capping material directly onto the pulp tissue to maintain long-term pulp vitality and function. After an initial inflammatory reaction, a new generation of odontoblasts is required to initiate pulp/dentin complex regeneration (1). This process requires the presence of dental pulp stem cells (DPSCs) and their migration to the injury site, where they regenerate reparative dentin (2, 3). Pulp regeneration success depends on the degree of pulp inflammation and the presence of regenerative signals. Recent studies have shown the involvement of complement activation after carious lesions, both in the control of inflammation and the initiation of the dentin-pulp regeneration process (4, 5).

Indeed, the complement system is a powerful and highly regulated protein cascade of innate immunity (6–8) that can be activated by traumatic injuries or infectious agents and plays a key defensive role. Its activation leads to the production of anaphylatoxins (C3a, C4a, and C5a) involved in the recruitment of immune cells (9), the production of potent inflammatory mediators, and the formation of the cytolytic membrane attack complex (7, 10, 11).

Under bacterial stimulation, pulp fibroblasts have been shown as the first nonimmune cells that can secrete all complement components, such as bioactive C3a, C5a, and the membrane attack complex (12). These molecules seem to play a major role during the early steps of pulp regeneration. C3a is involved in pulp fibroblasts and DPSC proliferation, DPSC mobilization, and specific recruitment of pulp fibroblasts (13). C5a has been shown to be involved in the specific recruitment of DPSCs (4, 14, 15), nerve sprouting by inducing nerve growth factor secretion by pulp fibroblasts (16), and macrophage and monocyte recruitment (9). These data clearly demonstrate C5a involvement both in inflammation and regeneration within the pulp and explain our choice of analyzing C5a fragment effects in this study.

Biomaterials have been reported to activate the complement. Indeed, when their surfaces are exposed to body fluids, there is a rapid adsorption of various plasma

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proteins (immunoglobulin [Ig] G, C3, factor XII, and fibrinogen). This mechanism modifies the biomaterial surface and thereby activates the complement (17, 18). It has also been shown that biomaterials with OH, NH_3, or carboxylic acid groups are involved in complement activation (19).

The aim of this work was to investigate, *in vitro*, the influence of pulp capping materials on the recruitment of inflammatory and DPSCs via complement activation. The capping materials studied are Biodentine (silicate based), TheraCal (silicate and resin based), and Xeno III (a resin-based bonding system). An experimental model using injured dental pulp fibroblasts was developed to evaluate the effects of the materials on the recruitment of inflammatory cells and DPSCs, which are crucial steps of inflammation and regeneration, respectively. In the first step, secretion of the anaphylatoxin C5a by injured fibroblasts incubated in the conditioned media was evaluated. Next, we investigated whether exposure of these injured cells to conditioned media may influence the balance of recruitment of DPSCs required for regeneration or THP-1 cells involved in the inflammation.

Material and Methods

Reagents

Media, reagents, and cell culture supplies were purchased from Dutscher (Brumath, France). Primary antibodies were provided by R&D Systems (Lille, France) and Abcam (Cambridge, UK), and secondary antibodies Alexa Fluor were provided by Life Technologies (Saint-Aubin, France).

Primary Pulp Cell Culture

Primary pulp cells were prepared from immature third molars freshly extracted for orthodontic reasons in compliance with French legislation (informed patient consent and institutional review board approval of the protocol used) using the explant outgrowth method (20). Teeth were obtained from 3 different donors for each experiment (4 per donor). Pulp cells were cultured in minimal essential medium (MEM) as described previously (4).

Magnetic Cell Sorting

STRO-1 pulp stem cells were sorted from primary pulp cell cultures at passage 1 or 2 using mouse antihuman STRO-1 IgM antibodies coupled to magnetic beads according to the manufacturer's protocol (Dynal, Oslo, Norway) as described previously (4). After cell characterization, which was performed as described in a previous study (12), STRO-1–sorted cells will be referred to as DPSCs, whereas STRO-1–negative cells will be referred to as pulp fibroblasts (13).

THP-1 Cell Culture

THP-1 cells, a human monocytic cell line (Sigma-Aldrich, St Louis, MO), were cultured in Roswell Park Memorial Institute medium (RPMI) as described previously (21). Experiments were performed with phorbol myristate acetate–activated THP-1 cells (400 ng/mL, 24 hours).

Immunofluorescence Double Staining

DPSCs were grown in 8-well glass culture chambers. Subconfluent cells were fixed (70% ethanol for 20 minutes at 4°C), and nonspecific binding sites were blocked (1% bovine serum albumin in phosphate-buffered saline, 1 hour). Cells were incubated for 1 hour with mouse IgM antihuman STRO-1 (2.5 μg/mL) and mouse IgG antihuman C5aR (1 μg/mL) or isotypes (negative control). The cells were then incubated for 45 minutes with Alexa Fluor 488 antimouse IgM (2 μg/mL) and Alexa 594 antimouse IgG (2 μg/mL). THP-1 cells were grown in 8-well glass culture chambers. After fixation (2% paraformaldehyde, 20 minutes at 4°C), cells were washed (PBS), and the nonspecific binding sites were blocked (phosphate-buffered saline containing 1% bovine serum albumin). Cells were incubated for 1 hour with mouse IgG antihuman CD44 (1 μg/mL) or isotypes. Then, they were incubated for 45 minutes with Alexa 488 antimouse IgG (2 μg/mL). The cells were finally incubated for 1 hour with mouse IgG antihuman C5aR (1 μg/mL) and then 45 minutes with Alexa 594 antimouse IgG (2 μg/mL). Cells were counterstained with 1 μg/mL 4′,6-diamidino-2-phenylindole.

Preparation of Conditioned Media

The biomaterials were prepared according to the manufacturer's instructions in sterile calibrated silicone molds. Materials were incubated at 37°C for 6 hours to achieve complete setting. Samples were sterilized 15 minutes on each side using ultraviolet radiation and then incubated in MEM without serum for 24 hours to obtain the conditioned media. The contact surface between the material and the culture media volume was 0.05 cm^2/mL. The resulting media containing the biomaterials eluates will be called conditioned media.

C5a Quantification by Enzyme-linked Immunosorbent Assay

Pulp fibroblasts were cultured in 12-well plates. Subconfluent cells were washed and then injured (2 horizontal and 2 vertical direction lanes with a sterile scalpel). These fibroblasts were cultured in MEM or conditioned medium (1 mL, 30 minutes, 37°C). Intact pulp fibroblasts were also included in the assay. C5a was quantified by enzyme-linked immunosorbent assay in the supernatants in 96-well plates (Nunc Maxisorp, Dutscher) using the Duoset human C5a kit (R&D Systems) according to the manufacturer's instructions. All the experiments were performed in triplicate. Results are expressed as the percentage of C5a secretion of injured pulp fibroblasts cultured in MEM without serum (control).

Cell Migration

Cell migration assays were performed in 12-well plates (lower chamber) fitted with 8-μm diameter Boyden inserts (upper chamber). Pulp fibroblasts were cultured in the lower chamber at confluency (Fig. 1). After washing with MEM, they were injured and incubated either in MEM (control) or in conditioned medium (1 mL). Intact pulp fibroblasts in MEM were also included in the assays. The upper chambers were seeded with 100 μL DPSCs (10^4 cells/well) or

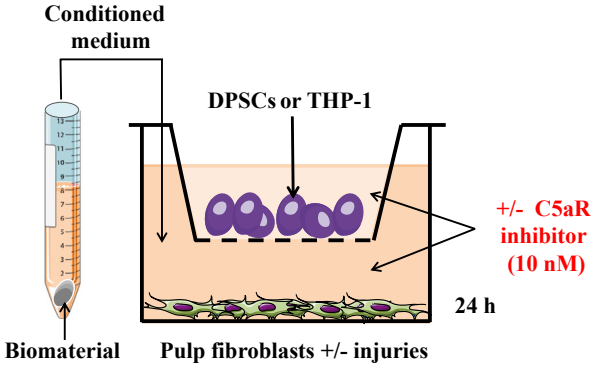

Figure 1. A schematic drawing of the cell migration protocol. Pulp fibroblasts were plated in the lower chamber, whereas DPSCs or THP-1 cells were plated in the upper chamber. Migration of DPSCs or THP-1 cells in function of injured fibroblast exposure to conditioned media was evaluated after 24 hours by counting the number of migrating cells. The same assays were performed by adding C5aR inhibitor (W54011) both in the upper and lower chambers.

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